Spermatophore production and sperm utilisation in the smooth newt *Triturus v vulgaris*

Thesis

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Spermatophore production and sperm utilisation in the smooth newt *Triturus v vulgaris*

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Abstract- This study investigates spermatophore production and sperm utilisation in the smooth newt *Triturus v. vulgaris*. Chapter one outlines the natural history of the species and describes the physiology of reproduction and the sexual behaviour of male and female smooth newts. Chapter two describes the general methodology.

Chapter three investigates the relationship between sperm accessory materials and spermatophore production. Spermatophore base height was correlated with male body size. The size of the glands that secrete the spermatophores was best predicted by body size, crest height and time post deposition.

Chapter four determines the gametic strategies exhibited by males. Males may be optimising their spermatophore output by depositing spermatophores of similar size and sperm content, but production may be limited by the availability of sperm and sperm accessory materials.

Chapter five examines the relationship between male body size and seasonal spermatophore production. Spermatophore production was correlated with body size, although crest height was the best predictor.

Chapter six investigates sperm utilisation in females. Female smooth newts may require courtship as well as insemination to induce ovulation and they may need to remate during the season in order to lay a full clutch.

Chapter seven investigates sperm utilisation due to polyspermy. Between 1 and 20 sperm may enter the ovum, although some ova were found to contain much higher numbers.

Chapter eight discusses the results from the preceding chapters. Male reproductive success may be limited by spermatophore production and by females' utilisation of sperm. Female reproductive success is related to an individual's fecundity, but this study has shown that it may be also partly determined by the number of mates obtained and the timing of insemination. Mating patterns in smooth newts may be influenced by females' requirements for sperm as well as by males' seeking multiple mates to enhance their reproductive success.

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Chapter 1. Introduction

This study focuses on two aspects of reproductive biology in the smooth newt *Triturus v. vulgaris*, namely, the production of spermatophores in males and the utilisation of sperm in females.

1.1. Introduction

Until recently, few studies have focused on patterns of sperm production in males because it has been assumed that all males exhibit a single strategy of 'inseminate as many females as possible' (Bateman 1948; Parker 1970). This view arose because sperm are regarded as cheap to produce, compared with large ova which incur high costs for females (Bateman 1948; Trivers 1972). Thus mating success by males is generally considered to be unconstrained by the production of sperm (discussed further in chapter 3).

This perspective changed in the light of Dewsbury's (1982) paper, in which he emphasised that sperm are rarely transferred in isolation but are transferred within ejaculates or spermatophores that contain accessory materials as well as sperm. These accessory materials may be costly to produce, even if sperm *per se* are cheap, and led Dewsbury to propose that the production of spermatophores or ejaculates may limit the reproductive potential of males. Sperm are usually transferred in large numbers during each mating, which may deplete the reservoir of stored sperm or accessory materials within a male and lead to a period of replenishment during which the male is unable to remate. Limitations on male reproductive potential may affect the timing of mating, the number of matings obtained during a breeding season, the likelihood of each mating leading to insemination of

the female and the number of sperm actually transferred during each mating. All these factors may affect male reproductive success, defined as the number of offspring reaching sexual maturity that are produced by an individual in a given time, either directly through the number of sperm transferred or as a consequence of the limitations on male mating success.

Physiological factors that affect the production of ejaculates or spermatophores in males may also affect the reproductive success of females with whom they mate. For example, although female reproductive success is determined primarily by the number of ova that each female produces (fecundity), constraints on the production of sperm or sperm accessory materials may limit the number of sperm transferred by a male during mating, resulting in only partial fertilisation of a female's clutch of eggs.

Similarly, extrinsic and intrinsic female factors may affect the reproductive success of both sexual partners. Such factors include the number and timing of matings obtained during the breeding season, the number of sperm taken up into the spermathecae (the tubules in which sperm are stored) during mating, the length of time that viable sperm are stored after mating and the efficiency with which sperm are utilised during fertilisation (these factors are discussed further in chapters 6 and 7).

These complex relationships, between the patterns of spermatophore production and sperm transfer in males and the patterns of mating and sperm utilisation in females, have co-evolved, as natural selection has favoured adaptations within members of each sex that influence the quantity and paternity of offspring produced by females in the population.

Trying to unravel these relationships is fraught with difficulty. Although various methods have been devised to collect sperm from species with external fertilisation, investigation of ejaculate production is difficult for species with internal fertilisation. The ejaculate is deposited within the female's reproductive tract, rendering it inaccessible except through flushing out or dissection, techniques that may underestimate the number of sperm transferred. Also, in some species intromission is not synonymous with ejaculation

(Dewsbury 1982). Although ejaculation can be induced artificially, ejaculates obtained in this way may not be representative of either the quantity or quality (measured as number of sperm) of ejaculates that are produced in response to receptive females (Baker & Bellis 1995). Only a few studies, such as the studies on sperm allocation in zebra finches *Taeniopygia guttata* (Pellatt & Birkhead 1994; Birkhead 1996) and in humans (Baker & Bellis 1995) have managed to collect entire ejaculates for quantitative measurement.

Spermatophore production in males and sperm utilisation in females have been investigated in many species of insects (Thornhill & Alcock 1983), but few studies have been carried out in urodeles, even though males also transfer sperm to females via spermatophores (discussed in chapter 4). Some aspects of spermatophore production in males have been studied previously in the smooth newt *Triturus vulgaris* (discussed fully in chapter 3), but to date no-one has undertaken quantitative measurements of the sperm or the accessory materials, or investigated the pattern of sperm utilisation in females. This species is very suitable for this type of study because the spermatophores can be readily intercepted before they reach the female's reproductive tract. Thus, not only can quantitative measurements be carried out on the spermatophores, but also the number of sperm masses transferred to each female and the timing of the matings can be manipulated.

In order to set the work in context and to be able to draw relevant conclusions from investigations of sperm production and utilisation in the smooth newt, it is necessary to briefly outline the life-history of the species.

1.2. Natural History of the smooth newt *Triturus v. vulgaris*

Populations of this member of the family Salamandridae are common in Britain and occupy both aquatic and terrestrial habitats, depending on their stage in the life cycle and the time of year. Adult and juvenile newts leave the breeding ponds between June and September and spend the next six months, terrestrially, in damp habitats, under logs and stones (Hagström 1982; Griffiths 1984) or buried in the soil (Verrell 1985). In spring, smooth newts migrate back to the breeding ponds, which may be temporary or permanent

bodies of water. Migration occurs during February and early March, and has been shown to be related to both temperature and rainfall (Harrison *et al.* 1983), or temperature alone (Verrell & Halliday 1985). At the end of the breeding season, many individuals remain in the ponds to feed, emigrating during late summer. A few adults and larvae that are too small to metamorphose may overwinter in the ponds.

In some populations of smooth newts, males are larger than females and in other populations there is no significant size difference between the sexes (Kalezic 1992). Although there is little size dimorphism between the sexes, newts exhibit sexual dimorphism during the breeding season; the cloaca of the male becomes enlarged and males develop a large dorsal crest which extends from behind their heads, along their backs and becomes indistinguishable from the rest of their tails (see Fig. 2.1). The crest facilitates oxygen uptake (see review in Houck & Sever 1994) and is also important in mate choice; female smooth newts preferentially pick up spermatophores from males with higher crests (Green 1991; Hosie 1992) and mate sequentially with males exhibiting higher crests than their previous mate (Gabor & Halliday 1997).

1.2.1 Physiology of gamete production

a) Sperm

The onset of reproduction in vertebrates that breed seasonally is fine-tuned each year by environmental cues that act on the sensory system of the individual (Moore & Deviche 1988) and stimulate the hypothalamic-pituitary-gonadal axis to release a cascade of hormones (see review in Houck & Woodley 1995). These hormones initiate mating and ensure that reproduction occurs when environmental conditions are favourable (Moore & Marler 1988; Wingfield & Kenagy 1991), for instance, enabling animals to delay the onset of breeding if the temperature remains low during the first weeks of spring.

In species that live in harsh and unpredictable environments or in environments with pronounced seasonal variations, where the opportunities for mating occur within a narrow time frame, reproduction may be constrained by physiological factors, such as the time

taken to mature gametes. If sperm development is not initiated until the environmental cues indicate that the conditions are favourable for reproduction, the conditions may be unfavourable by the time sperm development is completed. Spermatogenesis may make high energy demands on males, so that sperm development during the winter, which precedes the spring mating period in newts, would divert energy away from somatic maintenance and might thus affect survival.

Such constraints have led to the maturation of gametes and mating becoming separated, temporally (dissociated reproduction, *sensu* Crews 1987), and gamete production occurs after mating has ended for a current year, but before the onset of winter when food supplies are diminished. The mature gametes are stored until mating commences the following year. Smooth newts exhibit this strategy (Verrell *et al.* 1986), which means that males arrive at a breeding pond with a finite supply of sperm that cannot be increased during the current breeding season. It should be noted that male smooth newts may not exhibit a dissociated reproductive cycle in the strict sense of Crews (1987). Crews describes a dissociated cycle as one in which mating occurs when the levels of androgens in the plasma are low and the testes are not undergoing gametogenesis. Although gametogenesis is dissociated from mating in the smooth newt, there is evidence from *T. carnifex* (Zerani *et al.* 1991; Gobetti & Zerani 1992) that newts of the genus *Triturus* mate when their plasma androgen levels are high.

The annual cycle of sperm production in the smooth newt was investigated by Verrell *et al.* (1986) and is shown in Fig 1.1. A brief outline of the various stages of sperm production is given below (described fully in Lofts 1974; 1984; 1987; Moore 1987; Houck & Woodley 1995).

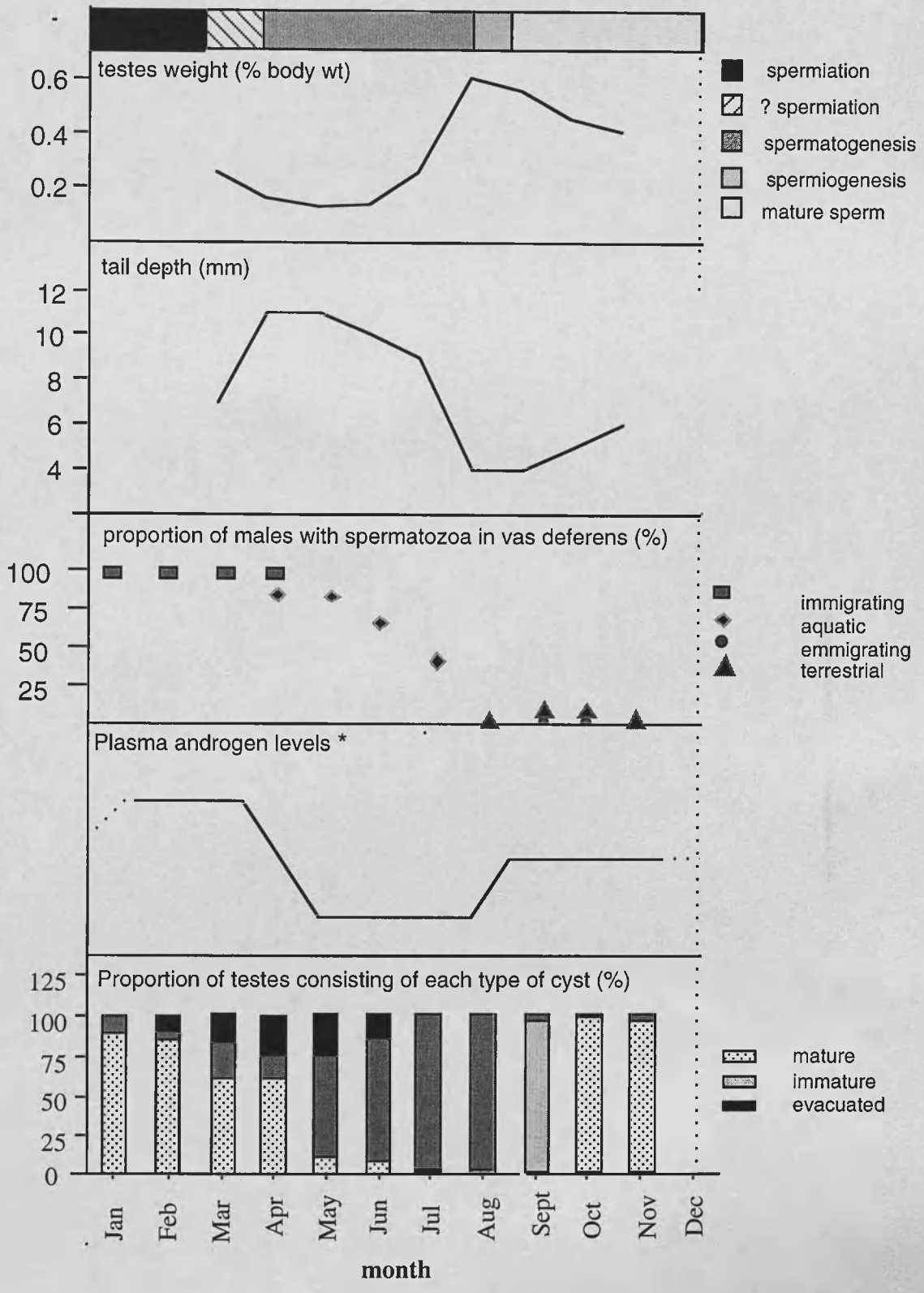


Fig.1.1 Reproductive pattern for *Triturus vulgaris* (*after Houck & Woodley 1995, using data from Verrell *et al.* 1986). For ease of reading, the labels for the vertical axes are located above the relevant data set.

Sperm are produced from primary spermatogonia within lobules (thin walled compartments) in the testes. The individual lobules are surrounded by interstitial boundary cells (analogous to Leydig cells) that synthesise androgens. Each primary spermatogonium is surrounded by flattened follicular cells (Sertoli cells), and, together, this group of cells is referred to as a germinal cyst. Urodele testes exhibit a zonal distribution of germinal cysts, whereby all the cysts within each zone are at the same stage of development.

At the end of the breeding season, each primary spermatogonium divides repeatedly to produce a clone of daughter cells, referred to as secondary spermatogonia. The secondary spermatogonia divide to form primary and then secondary spermatocytes, and finally to form elongated spermatids. Within a particular cyst, all the cells are identical in size and stage of development. Spermatogenesis refers to all the stages occurring within the germinal cyst, from primary spermatogonium to formation of spermatids.

At the end of the summer, the cysts containing spermatids rupture to release the Sertoli cell-sperm bundle into the lobule lumen (spermiogenesis). The sperm remain in the testes, surrounded by the Sertoli cells, throughout the winter. During the spring migration, possibly in response to the same environmental cues that initiate migration, the sperm are released and enter the vasa deferentia, via the efferent ducts (spermiation). All the spent Sertoli cells occupy the same region of the testes, which is referred to as glandular tissue (Lofts 1974; Verrell *et al.* 1986).

b) Ova

Oogenesis (development of ova) in female smooth newts begins during the summer, shown by an increase in the smallest size class of oocytes, after oviposition has been completed for that year (Verrell *et al.* 1986, Fig 1.2).

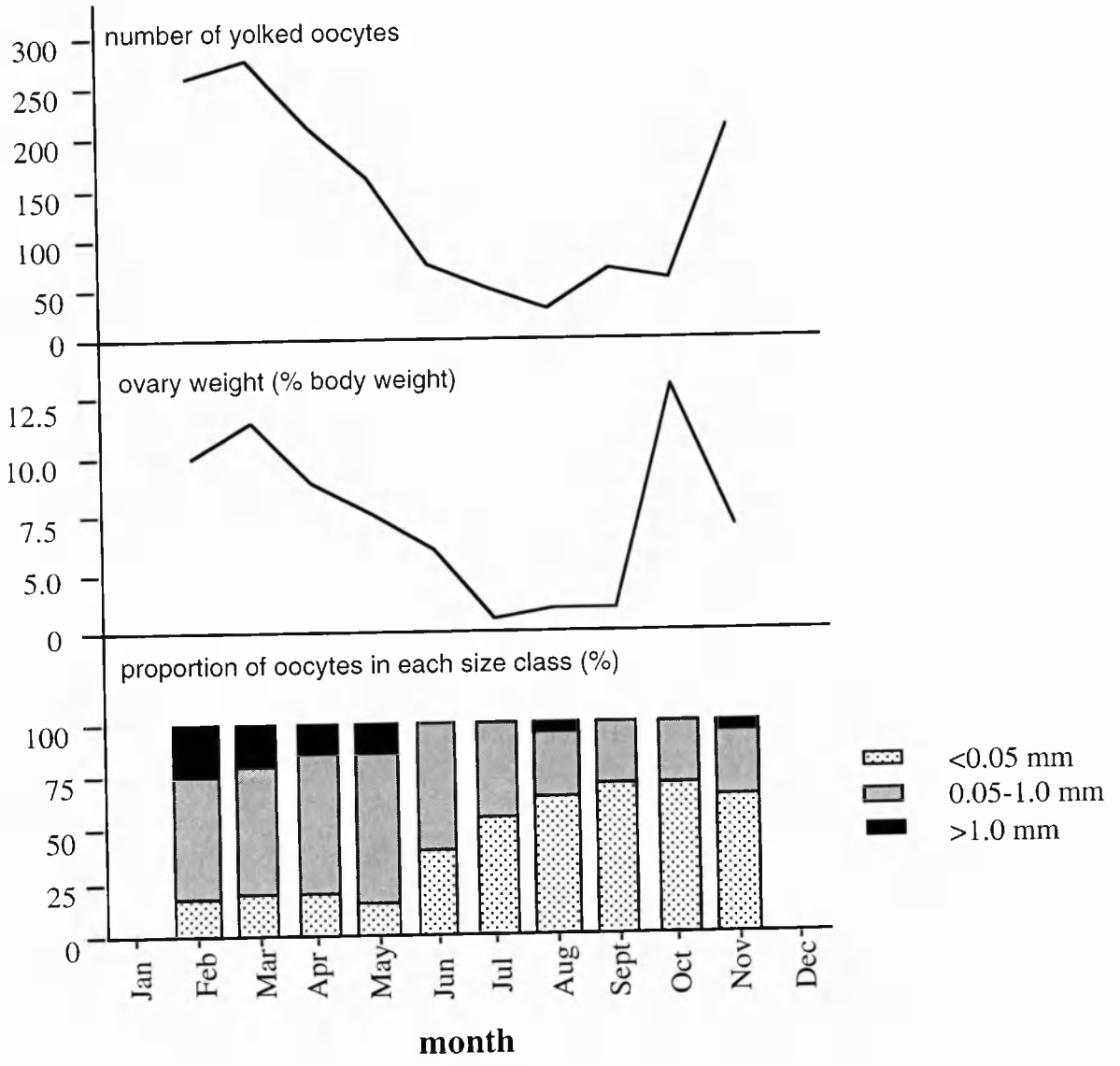


Fig. 1.2 Development of ovary and oocytes in the female smooth newt *Triturus vulgaris* (Data taken from Verrell *et al.* 1986). For ease of reading, the labels for the vertical axes are located above the relevant data set.

The final stages of preparation, namely, the incorporation of sufficient yolk into the oocytes for them to be ready for laying, shown by an increase in the largest size class of oocytes, occurs throughout the winter and may continue during the breeding season (Fig 1.2). Thus, unlike males, females exhibit an associated reproductive pattern (*sensu* Crews 1987). By undergoing vitellogenesis (production and incorporation of yolk into ova) during the winter, females may be able to allocate their stored resources differentially between oocytes and somatic maintenance (and thus survival) throughout the winter, in response to the environmental conditions pertaining at the time. Male and female smooth newts thus cease allocating energy to gamete production at different times of the year, probably because production and storage of mature sperm in males is less energetically demanding than the production and storage of large, yolk filled, ova in females.

1.2.2. Reproduction

Although fertilisation in smooth newts is internal, sperm are transferred from the male to the female externally, during an elaborate courtship (Halliday 1974, Fig 1.3). During the sperm transfer stage of the courtship sequence (Fig 1.3), the male deposits a spermatophore on the substrate and the female moves forward to pick it up in her cloaca. Typically, a courtship encounter consists of two or three sequences, up to a maximum of seven (Halliday 1974; 1975; Halliday & Houston 1978; Verrell 1986a; Baker 1990a; this study). Each sequence results in the deposition of a spermatophore. During a single encounter, a female may pick up between one to three sperm masses or fail to become inseminated.

Three to ten days after insemination (Pecio 1992; Hosie 1992; this study), females commence oviposition. Each egg is laid individually and wrapped in a leaf of pondweed. Female smooth newts take about five minutes to lay an egg, which is consistent with the findings of Diaz-Paniagua (1989) for female *Triturus marmoratus*, although female smooth newts can maintain the oviposition posture for up to fifteen minutes before swimming away (this study).

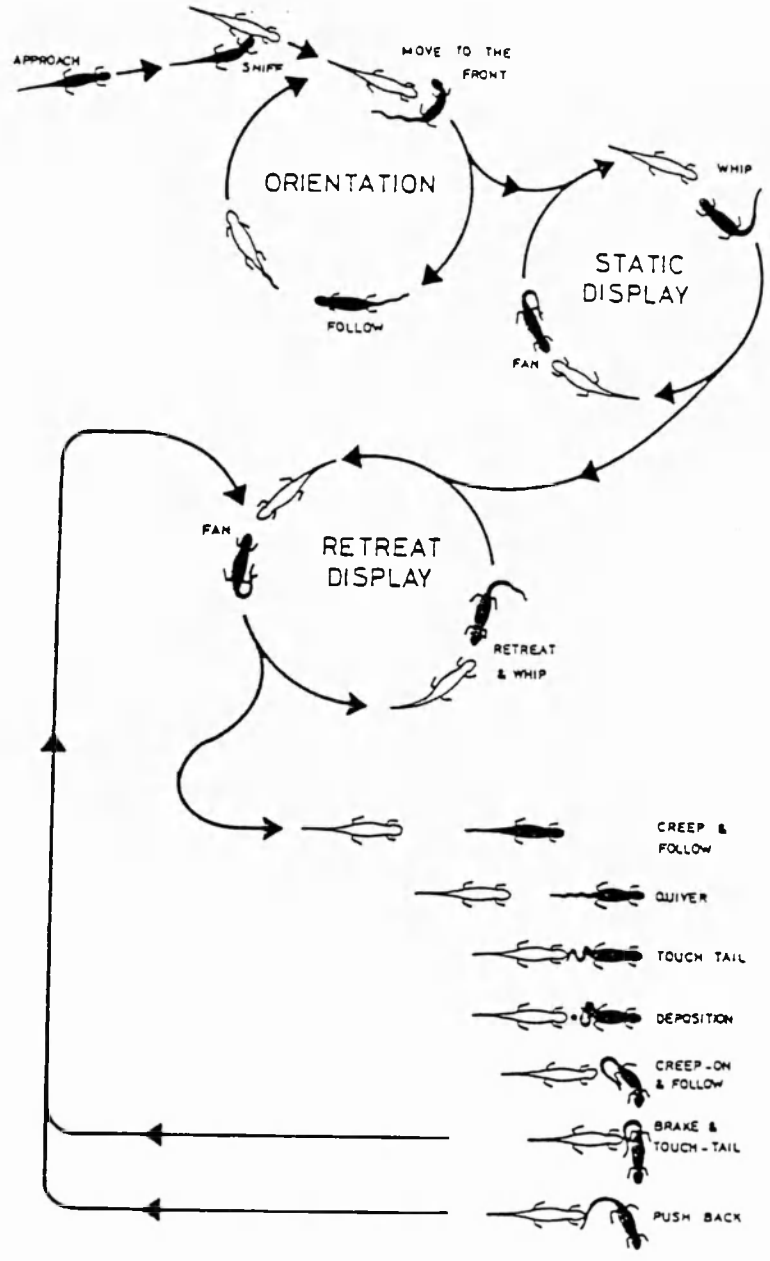


Fig 1.3 Schematic diagram of courtship display in male smooth newts (male=black, after Halliday 1977).

The time taken to lay each egg may expose females to predation, although the females remain motionless for much of the time which may minimise the risk. The laborious wrapping of each egg has been shown to increase the survival of eggs of *Triturus alpestris, cristatus* and *helveticus* in the wild (Miaud 1994) and to reduce the risk of predation on the eggs, especially from conspecific females (Miaud 1993; Gabor 1996) and water beetles *Acilius sulcatus* (Miaud 1993). Wrapping the eggs is thought to also supply the developing eggs with oxygen (Diaz-Paniagua 1989).

It has been known since the early seventies that male smooth newts, in the laboratory, can mate multiply (Halliday 1976), but recent laboratory studies have shown that female smooth newts also mate multiply, up to five times during a single breeding season (Hosie 1992). The level of multiple mating in female *Triturus vulgaris* in the wild is not known, but multiple paternity has been found in 94% of clutches laid by wild caught female *Triturus alpestris* during a single breeding season (Rafinski 1981). Hosie's study (1992) demonstrated that female smooth newts mate multiply, before the onset of oviposition and during the long oviposition period, which may profoundly affect their own reproductive success and the reproductive success of their partners.

1.3. Structure of the Thesis

Chapter 2 presents the experimental methods that are common to all the empirical studies. The first part of the thesis (Chapters 3, 4 and 5) investigates factors which may affect male reproductive potential. Chapter 3 investigates physiological factors that may limit spermatophore production in the male. Chapter 4 reviews the theoretical models currently proposed to explain how males allocate sperm to a series of spermatophores. These models are examined in the light of the mode of reproduction exhibited in the smooth newt. Chapter 5 investigates the prediction that larger males have higher reproductive potential and achieve higher reproductive success than smaller males. This hypothesis is tested in two ways, by looking at spermatophore production during one annual breeding

season, and by testing the ability of males of different sizes to mate multiply during a single reproductive episode.

The second part of this thesis (chapters 6 and 7) investigates sperm utilisation in females. Chapter 6 investigates the pattern of sperm utilisation under conditions of differential sperm supply and Chapter 7 investigates polyspermy in the ova of female smooth newts, enabling partial estimation of the efficiency of sperm utilisation at the site of fertilisation.

Chapter 8 discusses spermatophore production in the male and sperm utilisation in the female in relation to reproductive strategies and seasonal mating patterns. Reproductive strategies are a compromise between behavioural, physiological, and social factors that optimise the number of offspring produced by an individual in a given time (Duellman & Trueb 1994). The reproductive strategies of individuals determine the mating patterns of a population, and thus physiological factors that constrain spermatophore production and sperm utilisation in smooth newts may influence the mating patterns exhibited.

Chapter 2. Methods

Many of the materials and procedures used in this thesis are specific to a particular series of experiments and are described in the relevant chapters. This chapter describes methods that are common to all the experiments.

2.1. Collection of unmated newts

Adult males and females were collected on land during their annual spring migrations (1990-1995) to a breeding pond, approximately 12 m in diameter and less than one metre deep, in Milton Keynes, England. Usually, it is laborious to obtain terrestrial newts, but the pond at Great Linford is in the middle of a roundabout, enabling newts to be collected from the road on nights of high migration, which were identified by monitoring rainfall and temperature.

The adults are known to be unmated because courtship and insemination take place only during the aquatic phase. The sexes were segregated and placed into 'semi-aquatic' tanks containing both terrestrial and aquatic areas, enabling the individuals to determine the timing of entering the aquatic phase. Newts making the transition from the terrestrial to the aquatic phase undergo several adaptations, such as increasing the permeability of the skin (Smith 1951; Frazer 1989; Houck & Sever 1994). Consequently, it is important to allow the animals to undergo these changes before they enter the water.

The animals were collected for use in experiments in which the number of mates or the frequency of mating by particular individuals, during a breeding season, was under experimental control.

2.2. Collection of aquatic newts

Male and female newts, in breeding condition, were collected by hand-netting or trapping from two small ponds throughout each breeding season (1990-1995), for use in experiments which did not require knowledge of the previous sexual history of an individual. The pond used primarily is in the Conniburrow housing estate in Central Milton Keynes, and is approximately 10 m in diameter. The other pond is a small farm pond, less than 3 m in diameter, at Aspley Guise. Females were also collected regularly for use in the standardised courtship encounter trials (described below). The animals were only anaesthetised once, before being returned to their pond of origin, as repeated exposure to MS222 alters the hormone levels of newts (Giacoma pers comm) which might confound the results of the courtship trials.

2.3. Maintenance of newts

Male and female newts were housed in separate tanks within large tubs, two metres in diameter, and surrounded with water to buffer them from extremes of temperature. The exact size of the tanks, the complexity of the environment and the density of newts within them were determined by each particular experiment and are described in the relevant chapters.

Tanks for stocking newts, prior to selection into an experiment or for females used in the standardised courtship encounter trials, were 45x60x30 cm high, filled to a depth of 23 cm. The tanks contained gravel substrate, plenty of refugia (usually broken flowerpots), and pond weed, *Ceretophyllum demersum* and *Apium nodiflorum*, collected locally. Each tank also contained some bricks that protruded above the surface of the water. The bricks enable newts to climb out of the water, which is particularly important towards the end of the breeding season when the newts become terrestrial again.

The tanks were supplied with toad and frog tadpoles which graze on algae and thus control algal bloom. This is an effective method as newts do not prey on toad tadpoles, and only frog tadpoles that had attained a size larger than the gape size of adult newts were selected.

The density of newts within each tank varied up to a maximum of 20 animals. The tubs were situated outdoors and exposed to natural variation in temperature (3°C to 17°C, morning temperatures) and photoperiod. The tubs were covered with black mesh that reduces the light reaching the tanks by 85%. In addition to reducing light levels within the tanks and, therefore, reducing algal bloom, the mesh assisted heat retention during the cold months and heat dissipation in the warmer months. The newts were fed *Daphnia* and *Tubifex ad libitum.*

2.4. Observations and manipulations of courtship

The exact details depended on the nature of the experiment and are described in the relevant chapters.

2.4.1. Observation tanks

Unless otherwise stated, all experiments were carried out in observation tanks, 45x60x30 cm high, filled to a depth of 23 cm with aged tap water and containing a substrate of gravel, 5 to 6 mm in diameter. The tanks were kept at natural photoperiod and temperature, approximately one degree above that of the holding tanks, in an unheated shed (supplied with a pipe heater and thermostat to prevent the tanks from freezing in early spring). Prior to the experimental procedures, animals were placed in each observation tank and allowed ten minutes to settle. Previous studies had established that male smooth newts fail to respond to a standard courtship trial at temperatures below 7°C, and therefore experiments were not carried out on days when the temperature fell below this. Between experimental procedures the newts were returned to holding aquaria or individual tanks.

2.4.2. Identification of individuals

Individual newts were identified, where possible, using natural variation in colour and markings or the presence of deformities, such as missing digits or gaps in the crest and tail. Other individuals were toe-clipped to assist recognition. Each limb had only one digit removed and a maximum of two digits was removed from each individual. The number of

digits is restricted to ensure that their removal does not adversely affect male behaviour and survival (reviewed in Halliday 1996).

2.4.3. Morphometrics

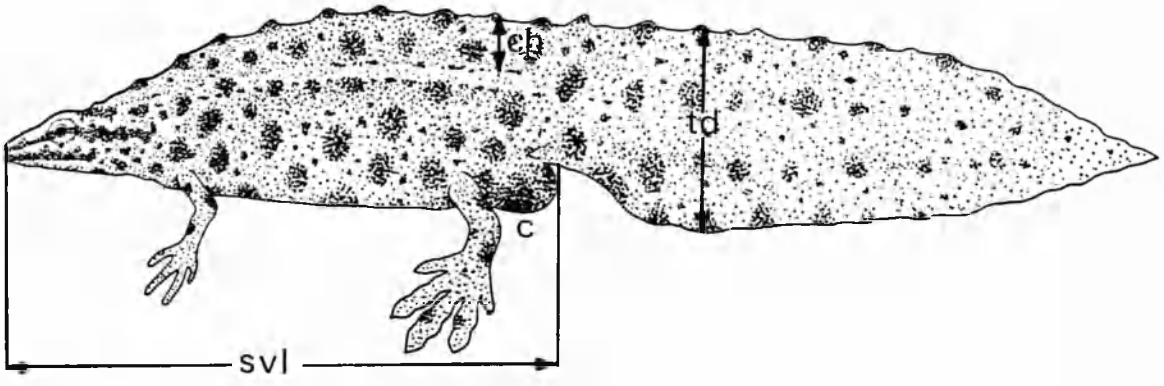


Fig 2.1 Male *Triturus vulgaris* showing positions from which the measurements were taken (snout-vent length [svl]; tail depth [td]; crest height [ch]). The position of the cloacal swelling (c) is also shown. Drawing after Halliday.

The following measurements were taken as described below (see Fig 2.1) and recorded for individuals of both sexes: snout-vent length [svl], tip of the snout to the caudal side of cloaca to the nearest 0.5 mm; tail depth [td], deepest part of the tail to the nearest 0.5 mm and weight [wt], weighed to 0.01 g precision using an electronic balance (g). Any water on the surface of the newt was allowed to drip off before weighing, but the animals were not dried as this may cause damage to the skin of fully aquatic newts. Weighing animals to this level of accuracy may be affected by any variation in the amount of water remaining on the skin and the individual's hydration level. However, variation in the hydration level of different individuals should be minimal in aquatic animals, and allowing excess water to run off prior to weighing should reduce variation from this source. Animals collected terrestrially were kept together in a moist environment for at least 12 h before weighing, in order to minimise any variation due to differential hydration. The animals were weighed in air so weight is used in this thesis to indicate the body mass of an individual. The body mass of an individual may be influenced by its body composition, which changes seasonally (Verrell *et al.* 1986), but this was not considered to be a problem in this study as all the animals

measured were in reproductive condition. In addition, male crest height [ch], highest part of the dorsal crest to the nearest 0.5 mm, was also recorded.

2.4.4. Anaesthesia

Newts were anaesthetised in MS 222-Sandoz (1 g 500 ml^{-1} tap water). The anaesthetised newts were washed under running water to remove surface traces of anaesthetic, which is particularly important prior to using females in standardised courtship encounter trials. Males that sniff unwashed, anaesthetised, strait-jacketed females lose interest in the female and cease courtship.

2.4.5. Standardised courtship encounters

The procedure for standardised courtship encounter trials was determined by Verrell (1986a), based on the manipulation of a female held in a strait-jacket (Halliday 1976). A female in good breeding condition, indicated by a deep tail and an egg-filled abdomen, was anaesthetised and placed in a strait-jacket consisting of a piece of split plastic tube, which fits around the newt's belly, mounted on a perspex rod. This device allows the female to be manipulated, by the experimenter, to mimic a highly responsive female and to elicit spermatophores from males. The experimenter can follow the male through several sequences of courtship display. Each encounter was terminated when the male reached sexual exhaustion; defined by a male failing to turn into creep, a stereotyped behaviour which initiates the spermatophore transfer portion of the courtship display, within two minutes after his last deposition or breathing ascent. Sexual exhaustion is also evident by the males' behaviour. The male may begin sniffing the female's cloaca, is more likely to go up to the surface of the water to breathe, and displays less vigorously on his return. This procedure enables comparison of various aspects of courtship and spermatophore production to be made between males, while controlling for variation in the responses of the female.

2.4.6. Pre-testing males for sexual activity

Several aspects of this study required males that were known to be sexually active. Therefore, males were pre-tested for response to straight-jacketed females, using the same

procedure as described for standardised courtship encounter trials. The pre-test was terminated after the male had executed some courtship display with his tail.

2.5. Returning newts to the wild

At the conclusion of the experiments, all newts, apart from the few males sacrificed in the cloacal gland study, were returned to their pond of origin. Some experiments generated several hundred to several thousand larvae and these were taken to the ponds from which their parents were obtained. The larvae had attained a large size and were close to metamorphosis. It is hoped that these larvae will assist in reducing any impact on the population, due to the temporary removal of a small proportion of the breeding adult population.

2.6. Statistics

Pearson product-moment correlation and stepwise multiple regression were carried out using JMP (a Spanish statistical package courtesy of Miguel Tejedo). Student's t-test was carried out using Excel and discriminant analysis was carried out using Statistica. All other analyses were carried out using SPSS.

2.6.1 Stepwise multiple regression

All the parameters, which are thought to contribute to the model, are put into an initial model and the multiple regression is run. The least significant parameter ($p>0.05$) is removed and the multiple regression is re-run using the parameters remaining in the model. These two steps are repeated until all the parameters in the model are significant ($p<0.05$).

2.6.2 Pearson Product moment correlations

Pearson product moment partial correlation coefficients are recorded as follows: $r_{[\text{score td; svl time}]}$. The two parameters, before the semi colon, indicate the two parameters which are being compared. Any parameters that are listed, after the semi colon, indicate parameters that are being held constant.

Chapter 3. Short-term physiological constraints on spermatophore production

3.1. Introduction

This chapter extends the discussion, initiated in chapter 1, concerning the relationship between gamete production and male reproductive success, and describes spermatophore production in the smooth newt.

3.1.1. Gamete production and male reproductive success

Reproduction is energetically expensive and, therefore, an individual's reproductive success will be constrained by any environmental or physiological factors that limit available reserves (Halliday 1987). The differential allocation of nutrients between reproduction and somatic growth and thus between current and future reproductive effort has been the subject of extensive study (Stearns 1992). However, until recently, male reproductive success has been considered to be limited solely by the number of mates an individual can inseminate, during a single breeding season or during a lifetime consisting of several breeding seasons, rather than by the number of gametes he can produce (Bateman 1948; Thornhill & Alcock 1983; Clutton-Brock 1988; 1991).

In order to inseminate as many females as possible, males should maximise the number of sperm that they produce (Parker 1970) and replenish their sperm supplies quickly after copulation (Trivers 1972). Thus males should invest minimally in each individual sperm (Parker 1970; 1982; 1984; Cohen 1973) while maximising the motility and survival of the sperm in the female reproductive tract. Males are also predicted to transfer sufficient sperm

in each insemination to saturate the female's reproductive tract or spermatheca, in which sperm are stored (Parker 1970), and to fertilise the full clutch of eggs (Knowlton & Greenwell 1984).

Another selective pressure that acts on male gamete production is sperm competition, in which sperm from different males compete within the female reproductive tract for access to ova. Parker (1970; 1984; 1990) describes sperm competition as a raffle, in which the male with the most sperm in the female reproductive tract will sire most offspring. Empirical studies have provided evidence in support of this theory, e.g. in chickens (Martin *et al.* 1974). The role of sperm competition in shaping male gametic strategies is further discussed in Chapter 4.

Taken together, the selection pressures discussed above will favour the production of many tiny sperm (Parker 1970; 1982). Thus these early theoretical models concluded that sperm are cheap to produce and, therefore, that male reproductive success is not limited by sperm production.

3.1.2. Physiological constraints on gamete production

As highlighted in Chapter 1, Dewsbury's (1982) paper on ejaculate cost and mate choice changed the perspective on the costs of gamete production. Dewsbury proposed that ejaculates may be costly to a male and may limit male reproductive success, comparable to costs of gamete production in females limiting female reproductive success. Recent empirical work has confirmed that male reproductive success is limited in some species by either the rate of production of sperm, e.g. in the lemon tetra *Hyphenobrycon pulchripinnis* (Nakatsuru & Kramer 1982) and in *Drosophila* (Pitnick & Markow 1994) or accessory materials, e.g. in butterflies (Svärd & Wiklund 1991). Dewsbury suggested that successive ejaculates may contain significantly reduced numbers of sperm, due to physiological mechanisms constraining the production of sperm or sperm accessory materials. The effect of these constraints on male gametic strategies is investigated in Chapter 4. Dewsbury also proposed that the transfer of large numbers of sperm in each ejaculate may deplete the

amount of sperm or sperm accessory materials stored in the male, generating a recovery period before the male can again deposit a large number of spermatophores. The requirement to replenish sperm or sperm accessory materials may limit male mating success, especially in species in which males may deposit several spermatophores during a single mating and have to replenish their supplies of spermatophores before they can inseminate another female, e.g. in smooth newts (Verrell 1986a), or in which large amounts of nutrients are also passed to the female, e.g. in butterflies (Svärd 1985; Svärd & Wiklund 1986; Svärd & Wiklund 1989).

3.1.3. Spermatophore production in the smooth newt

The spermatophore

The role of the spermatophore in sperm transfer in urodeles has been known since the Eighteenth Century (Spallanzani 1780 and Rusconi 1821, cited in Halliday 1974; Robin 1874, cited in Sever & Houck 1985). Spermatophores of newts that breed aquatically consist of a sperm mass supported on a gelatinous base (Zalisko *et al.* 1984; Sever 1992, Fig 3.3). The postulated function of the spermatophore base is to raise the sperm mass above the substrate, and thus facilitate pick-up of the sperm mass by the female's cloaca (Halliday 1974). During spermatophore deposition and transfer (described in Chapter 1), the sperm mass can be seen by the naked eye as a white elongated structure, 3 to 4 mm long. Successful pick-up of the sperm mass by the female occurs when the sperm mass is in this configuration. Within a few minutes of deposition, the sperm mass becomes spherical and will no longer adhere to the cloaca of the female. Occasionally, the sperm mass remains attached to the male's cloaca, which makes it impossible to locate the gelatinous base on the substrate.

Formation of the spermatophore

The spermatophores of urodeles are composed of mucopolysaccharides, also termed glycosaminoglycans, and glycoproteins, which are secreted by glands within the cloaca of the male. Studies of several species of urodeles have revealed that the spermatophore base is

moulded in the cloacal lumen. For example, in males of the *Ambystoma jeffersonianum* complex, the sets of horns forming the central depression at the top of the spermatophore base correspond to obvious features of the cloaca (Uzzell 1969). A histological study by Sever and Houck (1985) verified the formation of the spermatophore within the cloaca of male *Desmognathus ochrophaeus*.

Cloacal anatomy

The cloacal anatomy of salamanders was first described in *Triturus cristatus* by Heidenhain (1890). The following brief description of the internal cloacal anatomy of male *Triturus vulgaris* is taken from Sever *et al.* (1990). The cloacal swelling lies on the ventral surface of the newt, adjacent to and posterior to the hind legs (see Fig 2.1, Chapter 2). Inside the cloacal swelling lies the cloacal chamber through which faeces, urine and reproductive products are vented. The posterior portion of the intestine, the urogenital ducts and the bladder merge to form the cloacal tube (Figs 3.1 and 3.2 A-C). The majority of the space in the cloacal chamber is filled with the pseudopenis, which is attached to the roof of the cloacal chamber by a narrow band of tissue (Figs 3.1 and 3.2 C-D). The internal surface of the cloaca is lined with glands that secrete the base of the spermatophore and the matrix for the sperm mass. The spermatophore base is formed in the cloacal chamber, around the pseudopenis, which is why the base is hollow. The horns of the spermatophore base are formed by the invaginations of the cloacal chamber (Fig 3.2 E) and the sperm mass is formed in the cloacal tube.

Annual cycle of cloacal gland activity

During the summer, the cloacal glands of male smooth newts regress and hypertrophy is initiated in the autumn. When the newts become aquatic in the spring, the glands are enlarged ready for spermatophore production. In *Triturus marmoratus*, the cloacal volume has been shown to increase almost two-fold during the breeding season, due to an increase in the volume of the pelvic and ventral glands, but not of the dorsal and Kingsbury's glands (Romo *et al.* 1996).

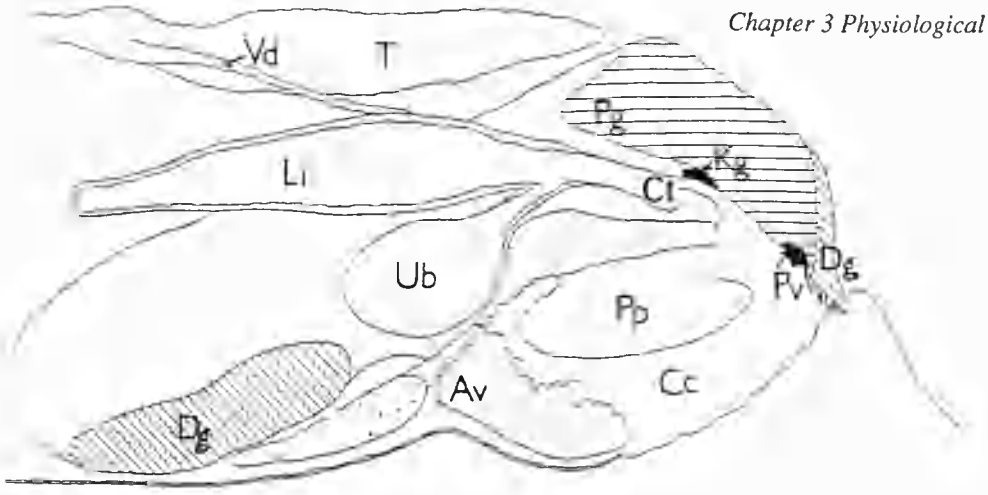


Fig 3.1. Drawing of the cloacal region of a male *Triturus vulgaris* showing the dorsal (Dg), Kingsbury (Kg), pelvic (Pg), anterior ventral (Av) and posterior ventral (Pv) glands. The positions of the large intestine (Li), the urinary bladder (Ub), the pseudopenis (Pp), the vas deferens (Vd), the testis (T) and the cloacal tube (Ct) are also shown. Cc=cloacal chamber; I=cloacal invaginations. (Adapted from a drawing by Tim Halliday, which was based on a series of histological sections prepared by Paul Verrell).

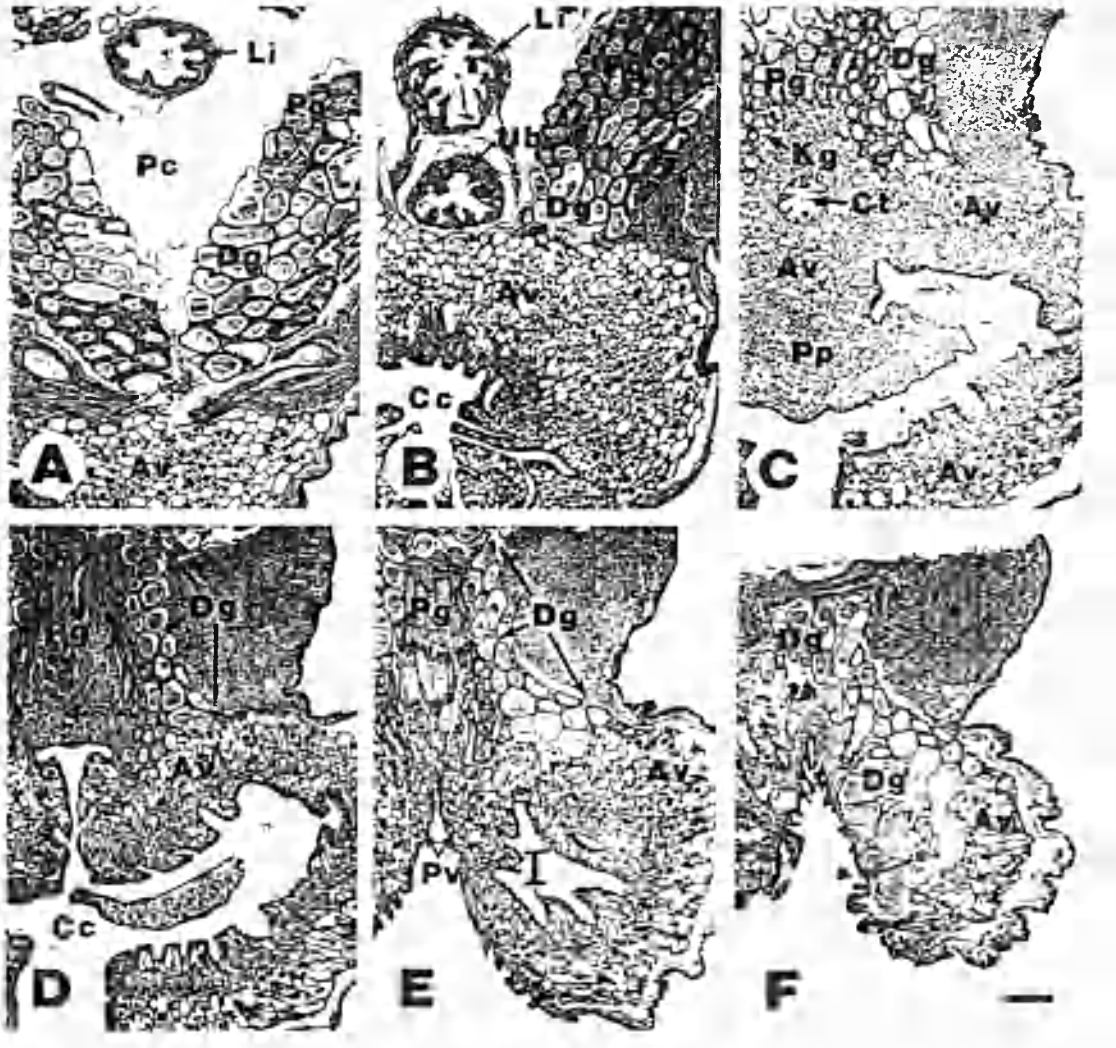


Fig 3.2. Transverse sections through the cloaca of a male *Triturus vulgaris* showing the cloacal glands (Figure reproduced from Sever *et al.* 1990, annotation as in Fig 3.1).

In *T. vulgaris*, secretory products are present in the glands throughout the year, but the glands are only full in May to July (Sever *et al.* 1990), which is unexpected as courtship and insemination commence in March or April. However, this conclusion may be inaccurate, as only one male, exhibiting depleted glands, contributed the data in April. One explanation for the depleted glands is that the male may have mated recently, which agrees with the finding that most of the sexual activity in the wild occurs at the beginning of the season (Verrell & McCabe 1988). Alternatively, males do not exhibit full glands while still terrestrial in the spring (Sever *et al.* 1990), suggesting that increased synthesis of cloacal gland secretions may be triggered by immigration to the aquatic environment. Therefore, this 'April' male may have recently entered the water and increased synthesis of secretory products in the glands may not yet be evident.

Structure of the cloacal glands

The cloacal glands of smooth newts comprise the dorsal, pelvic, ventral and Kingsbury glands (terminology standardised by Sever 1981), which can be clearly distinguished by their relative positions, morphology and histology (Sever *et al.* 1990). Each type of gland comprises many tubules; each tubule consists of secretory (epithelial) cells that line the tubule lumen in which the secretory products are stored. In *Triturus marmoratus*, the diameters of the tubule lumens of the pelvic and ventral glands are larger during the breeding season than in the summer, indicating storage of secretory products, but the heights of the epithelial cells lining the tubule lumens do not vary throughout the annual cycle (Romo *et al.* 1996).

The ducts of the tubules empty into the cloacal chamber and have been clearly demonstrated in *Salamandrina terdigitata* (Brizzi *et al.* 1990) and *Salamandra s. gigliolii* (Brizzi & Calloni 1992). The position of the ducts within the cloaca aids in attributing the secretion within the tubule, and thus the gland, with a particular portion of the spermatophore. The relationship between the various glands and the parts of the spermatophore has been determined in *Desmognathus ochrophaeus* (Sever & Houck 1985), but it is not known what causes the different secretions of the cloacal glands to gel together to produce the spermatophore. The secretions are neutral, sulphated and non-sulphated glycosaminoglycans, which are known

from studies in animals to swell on contact with water. Some of the secretions also contain proteins and it is known in animals that some proteins in the extracellular matrix, called selectins, bind to specific carbohydrates and thus enable cells to adhere to each other (Dow *et al.* 1996). Thus the secretions may interact with the pond water, and with each other, as the male's cloaca opens, forming the spermatophore independently of any enzymatic action.

Each tubule is surrounded by a layer of myoepithelial (muscular) cells that respond to neurotransmitters and release the secretions from the tubules (Hardy & Dent 1983). Local intramuscular injection of norepinephrine causes emission of 'typical' sperm masses which are not attached to spermatophore bases, and injection of either acetyl choline or norepinephrine causes secretions to be released from the anterior ventral glands. The inability to elicit complete spermatophores by local injection of neurotransmitters suggests that the release of the different neurotransmitters, which occurs during spermatophore formation, is coordinated by the nervous system.

Dorsal gland

The dorsal gland secretes pheromone that attracts females (Malacarne & Vellano 1987), assisting the males to initiate and maintain courtship encounters. A recent study (Brizzi *et al.* 1995a) has found that, in salamanders which mate aquatically, the ducts from the dorsal gland secrete into the cloaca and pheromones are transported to the female by movements of the male's tail, whereas, in terrestrial salamanders, the ducts open externally to the cloaca, facilitating transfer of the pheromone to the ground. The pheromone has been characterised in a few species, namely, in *Triturus carnifex* as a progesterone-like substance (Belevedere *et al.* 1984), in *Triturus marmoratus* as a neutral glycoprotein containing alpha-N-acetyl galactosamine (Romo *et al.* 1996), and as a decapeptide, sodefrin, in *Cynops pyrrhogaster* (Kikuyama *et al.* 1995). Sodefrin, extracted from dorsal glands of male Japanese newts or synthesised in the laboratory, attracts conspecific females, but has no effect on heterospecific female *Cynops ensicauda,* suggesting that pheromones are species-specific and may be important in species recognition. In smooth newts, the dorsal gland may weigh up to 10% of an individual's body weight (Verrell *et al.* 1986), indicating that pheromone production is

a major component of male reproductive effort. The replenishment of secretory products in the dorsal glands is outside the scope of this study.

Pelvic and Kingsbury glands

The ducts from the pelvic and Kingsbury glands secrete into the cloacal tube (Sever *et al.* 1990), suggesting that these glands (Figs 3.1 and 3.2 C) secrete the matrix of the sperm mass. Secretions from the pelvic glands are neutral glycoproteins (containing beta-N-acetyl galactosamine in *Triturus marmoratus*, Romo *et al.* 1996), that stain intensely PAS positive (Sever *et al.* 1990), which corresponds with the PAS staining of the sperm mass (Zalisko *et al.* 1984). The glycoproteins may give the matrix of a sperm mass some rigidity, which may be important in the transfer of sperm masses to the cloaca of a female during mating.

Ventral glands

The ventral glands comprise two groups, the anterior and posterior ventral glands, which are histologically distinct. The anterior ventral gland forms the swollen cloacal region characteristic of a male newt in breeding condition (Figs 2.1 and 3.1). Secretions from the anterior ventral gland are stained intensely with PAS, indicating carbohydrates (Sever *et al.* 1990), but so far the sugar moiety has not been identified in any species (Romo *et al.* 1996). Current studies suggest that the secretions of the ventral glands in salamandrids do not contain protein (Sever 1992), so the rigidity of the spermatophore base must be a consequence of the interaction of the glycosaminoglycans with the pond water. In mammals the shape of the eyeball is maintained by glycosaminoglycans, which react with water to produce a viscous gel that is very resistant to compression (Dow *et al.* 1996).

The spermatophore base also stains intensely with PAS (Zalisko *et al.* 1984) and recent studies have shown that the ducts of the anterior ventral glands secrete into the cloacal cavity and around the pseudopenis (Sever *et al.* 1990), confirming that secretions from these glands form the spermatophore base. Secretions from the posterior ventral glands and the pelvic glands attach the sperm mass to the spermatophore base (Sever *et al.* 1990), and may enable the sperm mass to adhere to the female's cloaca during mating.

3.1.4. Physiological constraints on spermatophore production in the smooth newt

Halliday (1987) has previously drawn attention to the fact that, in male smooth newts, spermatophore supply may be limiting and production costly. He describes physiological constraints on male reproductive success in *T. vulgaris* as operating on three time scales; long term (over a life-time, which is beyond the scope of this thesis), medium term (over a breeding season, investigated in chapter 5) and short term (during a courtship encounter or between encounters, investigated in this chapter).

Constraints on spermatophore production during one encounter

The energetic costs of producing one spermatophore base have been estimated in *Desmognathus ochrophaeus* as 3.51 J or 1% of a male's daily ingested energy (Marks & Houck 1989) which exceeds the energy expended on courtship by a courting pair (2.70 J, Bennett & Houck 1983). These low levels of expenditure suggest that energetic constraints on spermatophore production are minimal, although these estimates did not take into account the high utilisation of sugar moieties in spermatophore production (discussed further in chapter 5). However, there is some evidence for limited cloacal gland capacity during single encounters in studies of the North American plethodontids. The cloacal glands of *D. ochrophaeus* are depleted after spermatophore deposition (Sever & Houck 1985), and males that are presented with a potentially receptive female for successive nights do not court every night (Verrell 1988). Similarly, Verrell (1986a) found that male *T. vulgaris* also require a 24 to 48 h recovery period before they are able to deposit the same number of spermatophores in a second test compared with the number deposited during the first test. These studies demonstrated that spermatophore production in newts is constrained, but they were unable to determine whether production is limited by depletion of the sperm accessory materials or by the number of sperm available in the vasa deferentia for packaging into sperm masses. Another study in *D. ochrophaeus* reported that the height of the spermatophore base and the volume of the sperm mass deposited in the last sequence of an encounter were reduced in size (Houck pers obs, cited in Marks & Houck 1989). This finding suggests that the

refractory period observed in male newts and salamanders, after courtship, may be due to the depletion and subsequent replenishment of secretory products in the cloacal glands.

3.2. Aims

The aim of this Chapter is to investigate the physiological constraints on short and medium term spermatophore production by focusing on production of sperm accessory materials. Section 3.3 investigates spermatophore base height during single encounters throughout the season. Section 3.6 determines the dynamics of the cloacal glands during the behavioural recovery period exhibited by males after courtship.

3.2.1. Hypotheses and predictions

The data obtained were used to test predictions generated by three hypotheses concerning spermatophore size and production in the male smooth newt (Table 3.1).

Table 3.1 Summary of hypotheses and predictions investigated in this study.

Hypothesis	**prediction**
Hypothesis: H_1 The spermatophore is moulded in the cloacal lumen.	*Prediction: 1* Spermatophore base height is determined by male body size, as larger males are known to have larger organs and glands than smaller males (Brody 1945, cited in Møller 1988a).
Hypothesis: H_2 Successful pick-up of sperm masses by females is related to the height of the spermatophore base.	*Prediction: 2* Spermatophore base height is independent of male body size and is maintained across an encounter and throughout the season.
	Prediction: 3 Spermatophore base height is related to male tail depth, as females are more likely to pick up spermatophores from males with deep crests (Green 1991; Hosie 1992).
Hypothesis: H_3 Spermatophore production is constrained by cloacal gland capacity.	*Prediction: 4* Spermatophore base height decreases, a) in subsequent sequences during an encounter and b) throughout the season.
	Prediction: 5 Spermatophore base height is maintained, a) in subsequent sequences during an encounter and b) throughout the season, but the number of spermatophores produced is limited.
	Prediction: 6 The period of reduced mating ability (the behavioural recovery period) exhibited by male newts, after courtship and sperm transfer, is a consequence of the requirement to replenish the secretory products of the pelvic and ventral glands.

3.3. Base height of spermatophores

3.3.1. Methods

Collection of newts from a breeding population

Male newts were collected throughout the breeding season in 1990 (as described in Chapter 2). Three or four times a week, newt traps were placed in the pond overnight and emptied the following morning. Ten females per week were also collected from the traps to use in standardised courtship encounters. Any females in excess of this number were put back.

Maintenance of newts

Each cohort was placed in a tank, 30x45x30 cm high, containing refugia and pond weed (as described in section 2.3). The tanks were placed in a cold room and maintained at a temperature of 12 to 14°C, and a photoperiod that mirrored the external conditions (adjusted weekly throughout the season).

Observation tanks

The observation tanks were 30x60x30 cm high, containing aged tap water to a depth of 23 cm at room temperature (16 to 23°C) and a substrate of gravel 5 to 6 mm in diameter. Several sizes of gravel and sand were tested for suitability for the collection of spermatophores (Table 3.2). Medium-sized gravel was found to be the most suitable substrate for quick and efficient removal of each spermatophore with least disturbance to the courting male.

Morphometrics

After testing for spermatophore production, morphometric measurements of the newts were taken (as described in section 2.4.3).

Table 3.2 Comparison of different substrates for suitability for the collection of spermatophores.

Substrate	diameter	spermatophore collection
large gravel	>6 mm	the spermatophore adheres to one stone, but the stone is difficult to pick up with forceps.
medium gravel	5/6 mm	the spermatophore usually adheres to only one stone and the stone can be lifted using forceps.
small gravel	<5 mm	the spermatophore often adheres to several stones. The weight of the stones tears the spermatophore when it is lifted in a wide-necked pipette.
sand	fine particles	the spermatophore can be collected using a pipette, but the particles of sand obscure the outline of the spermatophore making measurement difficult.

3.3.2. Production of spermatophores

Within two days of capture, the newts were placed overnight in a stock tank (described in section 2.3) in the laboratory with a natural photoperiod and a daytime temperature of 16 to 23°C. The next morning, one male was placed in each observation tank and allowed to settle for ten minutes. Spermatophores were elicited from the males using a standardised courtship encounter trial (described in section 2.4.5) and the total number of spermatophores deposited was recorded (spermatophore score). Each spermatophore was collected, individually, and coded for future identification, as described below. Each male was tested once for spermatophore production and then returned to the pond.

During each standardised courtship encounter, the experimenter manipulated the strait-jacketed female to mimic a highly responsive female until the male entered the sperm transfer stage. After tail-touch, while the male was depositing the spermatophore, the strait-jacketed female was removed from the tank. Once deposition of the spermatophore was completed, the male moved forward one body length, turned perpendicular into the position referred to as brake, and usually remained in brake while the spermatophore was collected. After collection of the spermatophore, the female was manipulated to approach the male again. The male always resumed courtship of the female on her return, although as the male completed more sequences he displayed to her at a slower rate.

While the male was in brake, the particular stone to which the base had adhered was identified. The stone with the spermatophore attached was lifted and placed gently into a submerged bottle. The sperm mass was removed and placed in a vial containing 1 ml distilled water, which was then frozen (-18°C) to await analysis. The base of a spermatophore is colourless and is rarely observed in the tank, but can be just seen, by the naked eye, when placed in a small volume of water. Thus for any comparative work to be undertaken the base needs to be stained. The spermatophore base was transferred to 0.4% formaldehyde and stored at room temperature until stained.

Staining

The bases of the spermatophores were stained initially using a modification of Luna's (Luna 1968 in Humason 1979) stain for mucopolysaccharides, in which the bases were stained with 0.1% alcian blue and then counter-stained with 0.1% neutral fast red (Fig 3.3). Unfortunately, counter-staining with neutral fast red appeared to shrink the bases slightly; the bases no longer wafted with water currents and appeared more dense. The outline of the spermatophore base could be clearly distinguished using alcian blue alone and, as the bases were being stained to facilitate measuring, counter-staining was deemed to be unnecessary. The optimum timing for staining with alcian blue and washing to remove excess stain was determined. The procedure adopted for staining the base of a spermatophore is described below and summarised in Table 3.3.

Table 3.3. Method developed to stain the base of a spermatophore prior to measurement.

STAINING A SPERMATOPHORE BASE
1) stain with 0.1% alcian blue for 2 min.
2) wash under running water for 4 min.
3) transfer back to 0.4% formaldehyde.
4) store until measured.

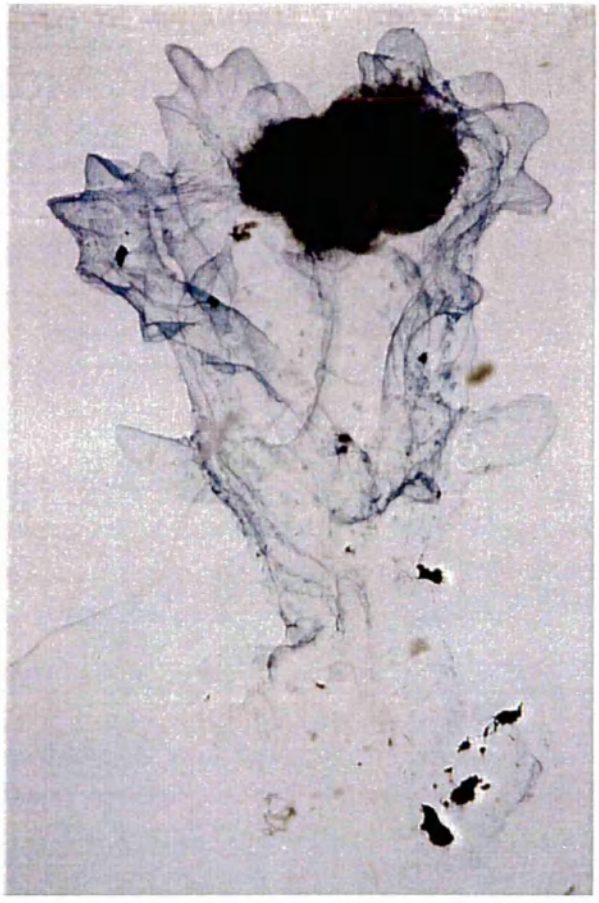


Fig 3.3 Photograph of a spermatophore of *T. vulgaris* showing the sperm mass attached to the base. The spermatophore is stained with alcian blue. Total height of the spermatophore=1.2cm.

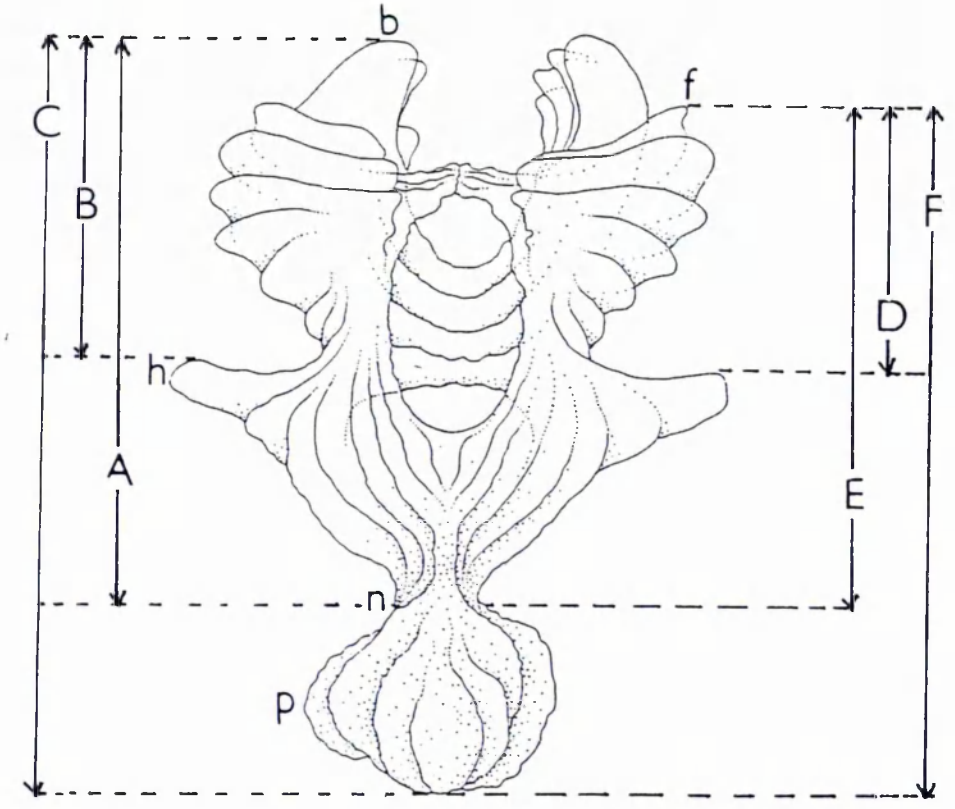


Fig 3.4 Drawing of a spermatophore base showing the positions from which measurements (A-F) were taken (b=apex of back of spermatophore; f=apex of front of spermatophore; h=horn; n=neck; p=portion which spreads out to adhere to the substrate). Adapted from a drawing by Tim Halliday, which was based on a spermatophore collected and stained by Verina Waights.

Measuring

The following technique was developed to measure the size of the spermatophore base. A spermatophore was transferred, via a wide-necked pipette to minimise damage, to a plastic petri dish (2.5 cm in diameter) containing distilled water. The dish was placed on a graticule marked in $1x10^{-2}$ cm and placed on a microscope stage. Placing the thin-bottomed dish on top of the graticule meant that the spermatophore base and the graticule could both be in focus at the same time. The base of the petri dish was thin and of uniform thickness and, therefore, any error in measurement was constant and would not affect comparative measurements between spermatophores. Several measurements of base height were taken as shown [Fig 3.4].

3.4. Results

3.4.1. Variation in spermatophore base height

There was a two-fold variation between the heights [measurements A-F in Fig.3.4] of the largest and smallest spermatophore bases (Table 3.4). Spermatophore base height is predicted to be determined by male body size, measured as snout-vent length and weight (prediction 1), and to be related to male tail depth (prediction 3) and time in season (prediction 4).

Table 3.4 Range and mean±s.d. of male body parameters, snout-vent length, weight and tail depth and of spermatophore base height (measurements A-F in Fig. 3.4, n=33).

		Range	mean± s.d.
Male body size parameters			
snout-vent length (mm)		42-51	46.1±2.74
weight (g)		1.75-3.22	2.46±0.46
tail depth (mm)		9-13	10.39±1.06
Spermatophore base height (mm)			
apex of back to neck	(A)	4.7-9.1	7.42±1.09
apex of back to horns	(B)	2.8-6.9	5.31±0.89
total back length	(C)	6.4-12.7	9.43±1.40
apex of front to horns	(D)	2.6-6.3	4.35±0.82
apex of front to neck	(E)	4.5-8.3	6.65±0.96
total front length	(F)	6-11.4	8.50±1.21

Step-wise multiple regression revealed that two measures of spermatophore base height were best predicted by weight and time in season, and three measures were best predicted by weight alone (Table 3.5). Step-wise multiple regression also revealed that spermatophore base height is not related to snout-vent length or tail depth (Table 3.5). The multiple linear regression models arising from the stepwise multiple regression are shown in Table 3.6. The regression model for measurement F includes time at p=0.07 (this parameter would normally be excluded from the model because p>0.05), as the relationship between measurement F and weight only shows a positive, but non significant trend (p=0.08).

Table 3.5. Stepwise multiple regression of measurements of spermatophore size (shown on Fig 3.4) and male snout-vent length [svl]; weight [wt]; tail depth [td] and time in season [time]. The table shows regression models after variables are removed from the equation, in turn, in order of least significance. Two sets of coefficients and p values are given: (i) taking into account all of the variables measured, and (ii) after stepwise regression including only those variables which are statistically significant (*p<0.05, n=33).

Measurement		**Male parameter**											
		svl		**wt**		**td**		**time**		**intercept**		**equation r^2**	**equation p**
		coeff	*p*	*coeff*	*p*	*coeff*	*p*	*coeff*	*p*	*coeff*	*p*		
A	**i)**	1.882	0.162	2.16	0.018	0.12	0.524	-0.06	0.022	16.35	0.003	0.30	**0.04***
	ii)			0.932	0.022			-0.050	0.036	9.187	0.000	0.24	**0.018***
B	**i)**	-0.083	0.52	1.245	0.100	0.065	0.69	-0.011	0.60	6.307	0.166	0.18	0.21
	ii)			0.793	0.018					3.35	0.0002	0.17	**0.018***
C	**i)**	-0.17	0.381	2.28	0.059	-0.12	0.94	--0.05	0.112	16.28	0.022	0.27	0.07
	ii)			1.23	0.024							0.16	**0.024***
D	**i)**	-0.169	0.239	1.226	0.15	0.082	0.65	-0.047	0.055	14.41	0.006	0.16	0.28
	ii)												
E	**i)**	-0.125	0.296	1.326	0.066	0.135	0.376	-0.027	0.175	7.66	0.07	0.19	0.18
	ii)			0.622	0.048					2.818	0.0008	0.12	**0.048***
F	**i)**	-0.184	0.29	1.936	0.067	-0.012	0.956	-0.051	0.079	16.55	0.009	0.24	0.08
	ii)			0.946	0.043			-0.05	0.067	10.21	0.0001	0.20	**0.04***

Table 3.6 Multiple linear regression models best describing the relationship between parameters of male body size and the height of the spermatophore base (measurements of base height shown in Fig 3.4; weight [wt]; time in season [time]= date expressed in Julian days).

Multiple linear regression model	r^2	significance
A=9.19+0.93**wt**-0.05**time**	0.24	p=0.018
B=3.35+0.79**wt**	0.17	p=0.018
C=6.33+1.22**wt**	0.16	p=0.024
E=2.82+0.622**wt**	0.12	p=0.048
F=10.21+0.95**wt**-0.05**time**	0.20	p=0.04

3.4.2. Spermatophore base height of successive spermatophores

To determine whether the height of successive spermatophore bases decreases during an encounter (prediction 4), spermatophore bases were placed into categories according to the ordinal position in which they were deposited. The heights of second and subsequent spermatophore bases were compared with the heights of bases in the first category, using repeated measures ANOVA (Fig 3.5). In this method, the height of a spermatophore base in a particular category was compared with the height of a corresponding spermatophore base in the first category, which had been deposited by the same individual during the same encounter. Thus each category did not need to contain the same number of spermatophores.

The heights of bases in the second and subsequent categories were not significantly different from the heights of bases in the first category (second spermatophore, $F_{(1,7)}=1.00$, p=0.35; third spermatophore, $F_{(1,4)}=1.25$, p=0.33; fourth spermatophore, $F_{(1,6)}=0.36$, p=0.57).

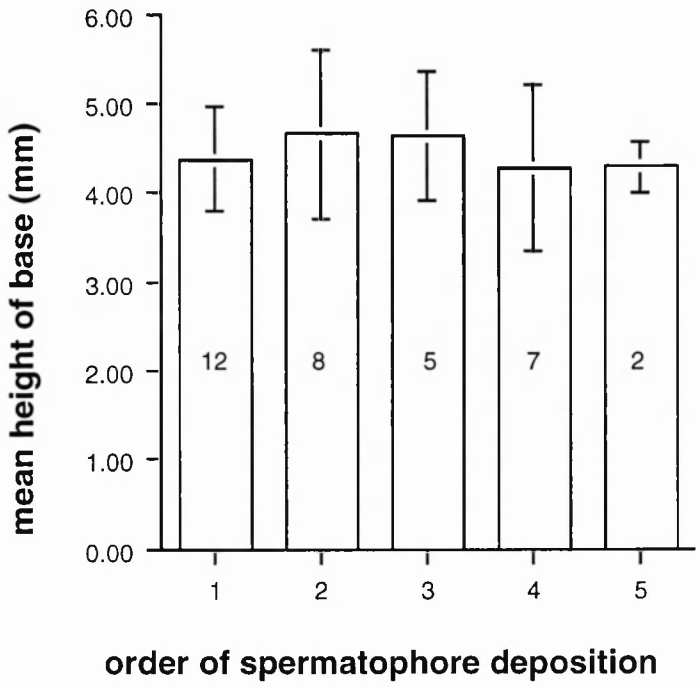


Fig 3.5 Comparison of spermatophore base height (mean ± s.d. of measurement E-apex of front to neck) of successive spermatophores deposited during one encounter. Numbers within the columns indicate the sample size for each category. The heights of bases in each category were compared to the heights of bases in the first category by repeated measures ANOVA. There was no significant difference between the heights of bases in subsequent categories compared with the heights of bases in the first category.

3.5. Discussion

3.5.1. Is the height of the spermatophore base correlated with male body size?

Stepwise multiple regression revealed that the height of the spermatophore base was best predicted by male body size (prediction 1), as indicated by weight, giving further credence to the proposal that the spermatophore base is moulded by the cloaca (Uzzell 1969; Sever & Houck 1985; Sever *et al.* 1990). Weight was a better predictor of spermatophore base height than snout-vent length suggesting that males with a higher body mass for their body length may have better developed cloacas than males with a lower body mass for their body length.

Similar relationships between spermatophore size and male body size are found in other species that transfer sperm via a spermatophore, e.g. in butterflies (Svärd & Wiklund 1986; 1989; Oberhauser 1988; Wiklund & Kaitala 1995) and in 42 species of bushcrickets (Vahed

& Gilbert 1996), suggesting that the organs that produce the sperm accessory materials are larger in larger males than in smaller males. It is not clear why measurement D (apex of front to horns) is so poorly correlated with male body size (Table 3.4). One reason may be that the horns curve up slightly, out of the plane of focus (see Fig 3.3), which may lead to measurement error.

3.5.2. Is the height of the spermatophore base constrained by cloacal gland capacity?

The heights of spermatophore bases, deposited during a single encounter, did not decline with successive spermatophore deposition. Thus the prediction that the height of the spermatophore base is reduced as the secretory products in the cloacal glands become depleted is not supported in this study (prediction 4). My finding that the height of the spermatophore base does not decrease with increasing order of deposition during an encounter contrasts with the observations in *Desmognathus ochrophaeus* (Houck pers obs, cited in Marks & Houck 1989). One explanation for this difference gives further support to the hypothesis that spermatophore bases are moulded in the cloacal lumen. In *Triturus vulgaris*, the majority of the cloacal chamber is occupied by the pseudopenis. Therefore, the ducts from the tubules of the ventral glands may secrete onto different portions of the lumen, preventing a fully-formed spermatophore from diminishing in size. The cloaca of male *Desmognathus ochrophaeus* does not possess a pseudopenis, so formation of the spermatophore base is unconstrained by structures within the cloaca. Consequently, as the glands become depleted in individuals of this family, successive bases may be smaller because they are composed of less material. This reduction in absolute base height may not affect reproductive success in male *D. ochrophaeus* because, unlike female *T. vulgaris*, the female desmognathids feel for the sperm mass with their cloacas.

In smooth newts, conservation of the heights of the bases deposited during an encounter does not preclude cloacal gland capacity constraining the production of sperm accessory materials; rather it implies that males cease depositing spermatophores when they run out of secretory products, supporting prediction 5.

Only one measurement of spermatophore base height was best predicted by time in season, suggesting that the influence of time in season may be slight. It should be noted, however, that this measurement of the spermatophore base decreased with time in season, which would be expected if the size of the cloaca decreased as the season progressed. The males in this study were collected sequentially throughout the season, thus any reduction in base height due to depletion of the cloacal glands may be masked if the males represented different cohorts arriving at the pond during the season.

3.5.3. Is the height of the spermatophore base related to successful pick-up of the sperm mass?

As discussed previously, the spermatophore base is thought to facilitate pick-up of the sperm mass by the cloaca of the female. Verrell (1982a) has shown that males prefer to mate with larger, more fecund females, which led Baker (1990b) to predict that males produce spermatophore bases that are an optimal size for pick-up by larger females. In this study, the height of the spermatophore base is positively related to male body size, so the prediction that the height of the spermatophore base is optimised to facilitate transfer of sperm to larger, more fecund females is not supported.

The finding that spermatophore base height is related to male body size suggests that, if the absolute height of the base determines whether successful sperm transfer occurs, smooth newts should show size-assortative mating. There is no direct evidence of assortative mating, but Verrell (1991a) demonstrated that female smooth newts prefer to remain in the proximity of male newts of similar body size. Although these two findings suggest that the height of the spermatophore base may be important in sperm transfer, pick-up success is not related to male body size in *T. vulgaris* (Halliday 1974; 1975; Hosie 1992). Thus females probably orient towards males of a similar size as part of the mechanism to identify conspecifics rather than as a consequence of mate choice. Pick-up success is related to male body size in the congeneric species, *Triturus cristatus* (Hedlund 1990), which may be a consequence of spermatophore size. However, this relationship is only observed at the beginning of the season when male crest height and tail depth have not reached maximum

values, suggesting that pick-up success is determined by factors other than spermatophore size.

The height of the spermatophore base is not related to the tail depth of male newts in this study, although the relationship between tail depth and pick-up success is well documented in two species of the genus *Triturus* (Hedlund 1990; Green 1991; Hosie 1992). This finding, in conjunction with the correlation between male body size and spermatophore size, suggests that the increased pick-up success observed from males with a large tail may be a response to other elements of male courtship, such as pheromone production or intensity of display.

Consequently, my findings, taken together with other studies, suggest that the spermatophore base produced by *T. vulgaris* males facilitates pick up of the sperm mass by ensuring that the sperm mass does not 'disappear' into the substrate, but that the absolute height of the base appears to be relatively unimportant.

3.6. Dynamics of the secretory cells of male cloacal glands

3.6.1. Methods

Collection and maintenance of newts

Male and female newts were collected and maintained as described previously (sections 2.2 and 2.3). The males were maintained for one week to minimise any effects due to previous mating and were subsequently tested over a four-day period to minimise any seasonal effects.

Morphometrics

Morphometric measurements of the newts were taken (described in section 2.4.3), and males between 47 and 52 mm snout-vent length (crest height: range 2-6 mm) were selected for the experiment to minimise body size effects. The weight of the males ranged between 2.11 and 3.89 g.

Depletion of cloacal glands

Male newts were placed in observation tanks (see section 2.4.1) and tested using a standardised courtship encounter trial (described in section 2.4.5). The number of spermatophores deposited during the encounter (spermatophore score: range 2-10) was recorded. At predetermined intervals (0, 1, 2, 8, 12, 24 h) post-encounter, the males were sacrificed by terminal anaesthesia (see section 2.4.4) and decapitation.

3.6.2. Preparation of the tissue

The tissue in this study was prepared by rapid freezing and was then sectioned using a cryostat. The method for preparation of the tissue is described below and is summarised in Table 3.7.

Fixation

The cloacal glands were isolated by two vertical cuts through the entire body, caudal and cephalic to the cloacal swelling (see Fig 2.1, Chapter 2), and fixed overnight in 20 ml of 4%

formaldehyde at room temperature (RT). Sectioning tissue at this stage resulted in the tissue tearing in the vicinity of the vertebra. The cloacal glands lie in close proximity to the vertebra, which means that the vertebra cannot be dissected out without the possibility of damaging some of the tubules. To avoid damage from either cause, the tissue was decalcified prior to freezing.

Table 3.7. The method for preparing the cloacal glands for measurement of the tubules is outlined below (see text for details). All procedures were carried out at room temperature unless otherwise stated.

PREPARATION OF CLOACAL GLANDS	
1)	Fix in 4% formaldehyde overnight.
2)	Decalcify in formic acid/formalin (Gooding & Stewart solution).
3)	Saturate in 20% sucrose for 4 h.
4)	Mount in 7% gelatine/20% sucrose (on ice) and leave overnight.
5)	Freeze in iso-pentane (on dry ice).
6)	Store at -80°C until sectioned.
7)	Section at $14x10^{-6}$ mm, in a cryostat at -22°C.
8)	Collect sections on chrome alum subbed slides.
9)	Stain with heamatoxylin & eosin.

Decalcification

Post-fixation, the tissue was decalcified (at RT) in 20 ml of formic acid/formalin (Gooding & Stewart solution in Bancroft & Stevens 1990). Every 24 h the solution was tested to determine if decalcification was complete. Equal volumes of the solution and saturated ammonium oxalate were mixed together (method of Milton Keynes Hospital, pers comm). The solution turns cloudy if calcium is still present and remains colourless when decalcification is complete. If further decalcification was required, the tissue was transferred to fresh solution. Tissue sectioned at this stage did not tear, but the sections were very friable and tended to collapse. This problem was overcome using a method developed for supporting spinal cord tissue (Stirling & Brown pers comm).

Sucrose and gelatine tissue support

The tissue was submerged in 20% sucrose (at RT for 4 h) and then placed into a mould (pre-cooled on ice) containing 7% gelatine in 20% sucrose. The mould was left overnight (at RT) in a damp box. The following morning the gelatine block was removed from the mould and plunged into iso-pentane (precooled on dry ice) for 30 seconds. The block was then maintained at -80°C until sectioned.

Sectioning

$14x10^{-6}$ mm transverse sections of the cloaca, from the caudal end, were cut using a Slee cryostat at -22°C. The sections were collected on chrome alum subbed glass slides and allowed to dry overnight (at RT).

Staining

The sections were stained with heamatoxylin and eosin (Harris in Bancroft & Stevens 1990) using a Linear Autostainer. Post-staining, the sections were preserved with DPX mounting medium (formulation Raymond A. Lamb) and cover-slips and allowed to dry overnight. An example of a stained section is shown in Fig 3.7.

3.6.3. Measurement and analysis

The diameters of the tubules were measured and analysed using the software program, Genias (Version 4.4, Applied Imaging 1992) on a Magiscan Image Analysis system. A section is manually viewed under a microscope and the image also appears on a computer screen. The Magiscan is calibrated for magnification and the image of the section is digitised. Lines drawn on the screen with a light pen will now correspond to an exact distance on the section (i.e. 1 pixel=$1x10^{-3}$ mm). Genias uses the calibration to calculate dimensions of the highlighted regions such as length, area, perimeter and diameter. The data were down-loaded into Word 4.0 and then imported into Excel 4.0 for analysis.

The perimeter of each tubule (Fig 3.8a) was drawn on the screen. Areas highlighted in this way could be selected and filled with solid colour and I used this feature to check that each

cross-section was representing only one tubule. The lines drawn around individual tubules may touch, resulting in the computation of one large cross-sectional area (CSA) for several tubules. This error generates false high values for the CSAs of the tubules and would, therefore, lead to the mean CSA for tubules in that gland being incorrectly calculated.

Measurement of the pelvic glands

Cross-sectional areas (CSAs) of the tubules were determined at various time intervals, after the encounter was terminated. The tubules are approximately circular in cross-section. However, the tubules twist as they traverse the cloaca, which may give rise to oblique cuts through the tubules, so that the tubules are elongated, not circular in cross-section (Fig 3.8a). Inclusion of the area of these elongated tubules would distort any estimate of tubule size, resulting in a comparatively high mean value. Genias can compute circularity of the area within the perimeter drawn on the screen, so this facility was used to exclude elongated tubules. The number of elongated tubules in several sections was established, by eye, and compared with the number of tubules, in the same sections, that Genias regarded as least circular. A perfect circle has a circularity value of 1.0. Excluding tubules with a circularity value less than 0.7 gave very comparable results with the number of tubules excluded by eye (Table 3.8).

Table 3.8. Excluding elongated tubules from the analysis. Examples of comparison of exclusion based on circularity, established using Genias, with exclusion by eye.

total number of tubules	**number of elongated tubules**	
	by eye	**by circularity**
128	16	16 with values <0.7
128	18	19 with values <0.7

Establishing the position of the tubules in the cloaca

The diameter of the tubules is not uniform throughout the cloaca (Sever *et al.* 1990). Therefore, it was important to establish that the tubules were being measured at the same position within the cloaca of each newt. The sections in Sever *et al.* (1990) were used to

establish boundaries between the different regions, designated A-E (Fig 3.4). In addition to the cloacal glands, the positions of the cloacal tube and the pseudopenis were noted. The centre of the pseudopenis is attached to the adjacent tissue, but at either end of the pseudopenis deep invaginations separate the two tissues (Fig 3.1). The portion of region C in which the pseudopenis is attached to the adjacent tissue was chosen for the comparative work, as this area can be clearly defined and readily identified in serial sections within and between newts, and contains tubules of both glands of interest (discussed in the introduction). Tubules of the pelvic glands were measured in eight sections selected to traverse region C (distance between selected sections = 98×10^{-6} mm). The mean CSAs increased slightly from the caudal to the cephalic end of the region, but the CSAs of tubules within each section varied considerably (Fig 3.6).

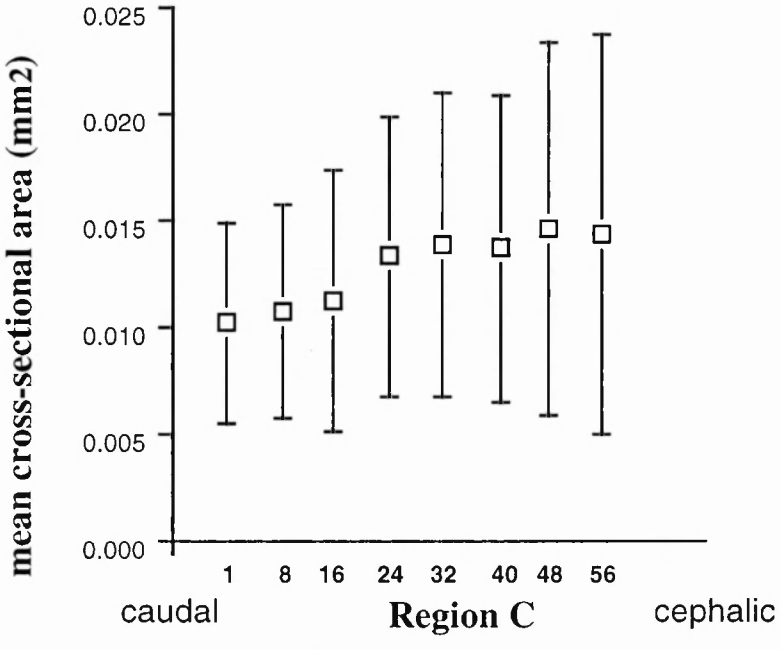


Fig 3.6. Mean cross-sectional area (±s.d.) of tubules in Region C of the cloaca of the smooth newt (Numbers on horizontal axis = the section number. Distance between selected sections=98×10^{-6} mm).

To ensure that these changes in CSA of tubules across the region did not mask any differences in CSA resulting from the experimental manipulation, the CSAs of tubules in the pelvic glands were measured in three positions of region C: i) post region B, ii) middle of

region C, iii) anterior to region D. The narrow range of body size used in this study should also minimise any effect due to individual variation.

Measurement of the ventral glands

The tubules of the ventral glands (Fig 3.7) are much smaller than the tubules of the pelvic glands, so a separate program was generated to measure these tubules using a calibration of 1.07 microns per pixel. Viewing the whole region of ventral glands resulted in an image too small for accurate measurement of the individual tubules. Therefore, four regions of tubules were measured as follows: the section was lined up so that the left hand side of the screen bisected the pseudopenis and the cloacal tube. All the tubules adjacent to the cloacal tube were measured. The procedure was repeated so that the right hand side of the screen bisected the pseudopenis and the cloacal tube, and the tubules in this new field of view were measured. The values for these two regions were combined and designated Field A (Fig 3.7b). The CSAs of tubules at the base of the cloaca were determined in the same way and designated Field B (Fig 3.7b). Unlike the tubules of the pelvic glands, the tubules of the ventral glands lie too close together to draw around the perimeter of each tubule (Fig 3.8b). Therefore, the CSA of each tubule was determined from the perimeter of the lumen, the inner edge of the secretory cells.

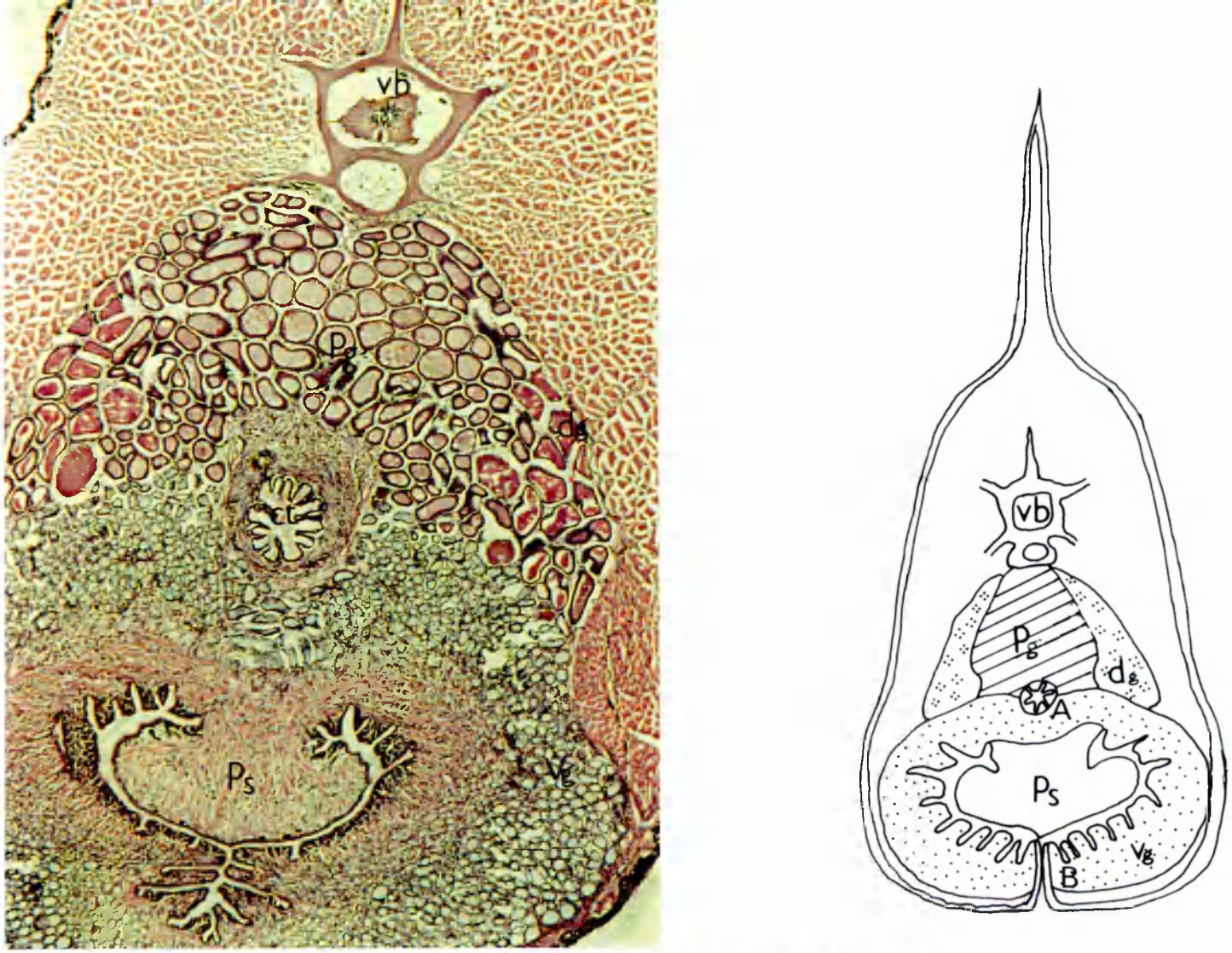


Fig 3.7a) A vertical section through the cloaca of the smooth newt in region C; b) drawing of vertical section through the cloaca in region C, showing position of the glands and the positions of the two fields [A and B] measured in the anterior ventral glands (see text for further details). Ps=pseudopenis; vb=vertebra; ct=cloacal tube; dg=dorsal glands; Pg=pelvic glands; Vg=ventral glands (anterior). Stained with haematoxylin and eosin (see text for details).

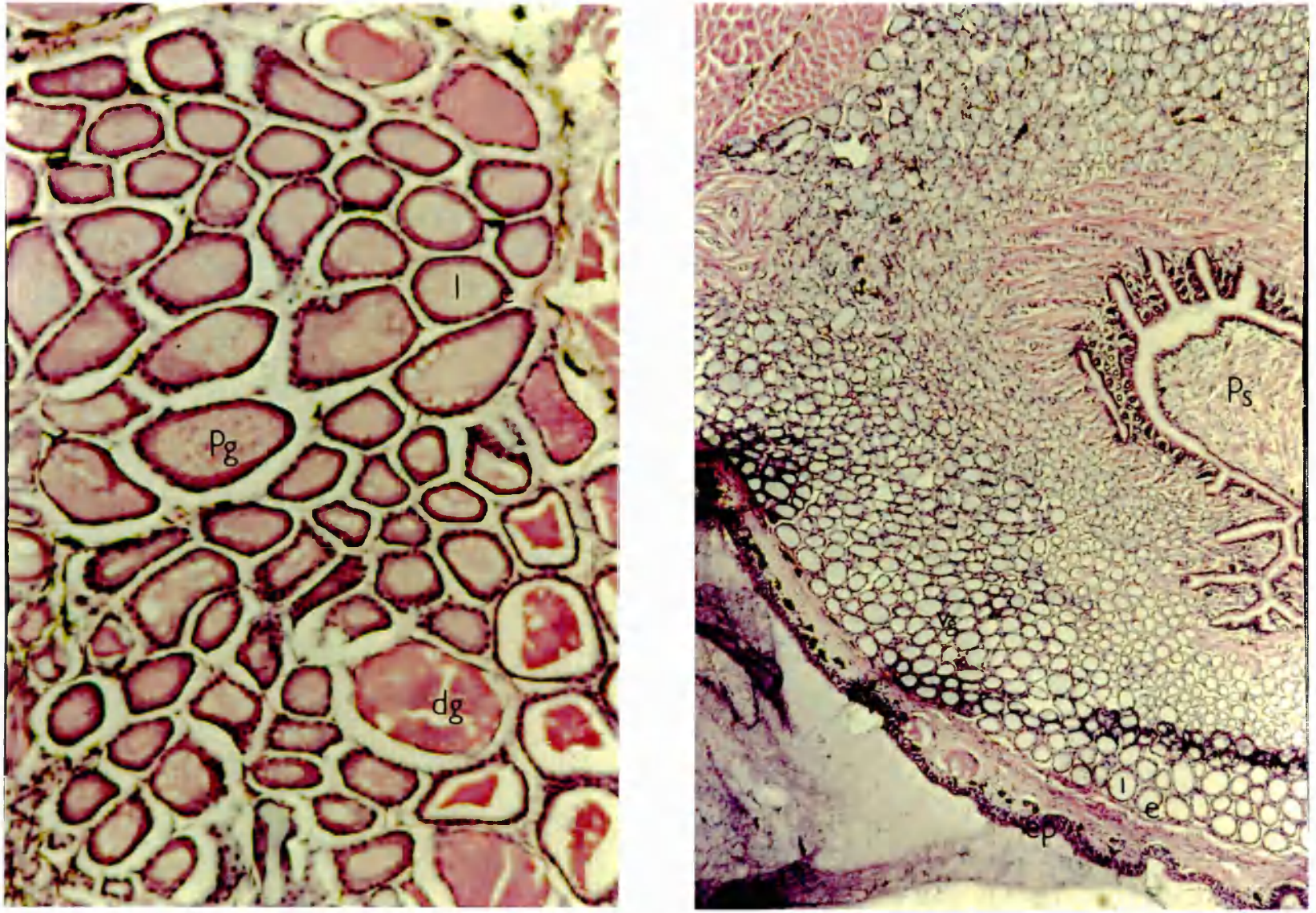


Fig 3.8 Vertical sections through a) tubules of the pelvic glands and b) tubules of the ventral glands (l=lumen, e=epithelial cells, ep=epidermis, Pg=pelvic glands, dg=dorsal glands; Vg= ventral glands; Ps=pseudopenis). Stained with haematoxylin and eosin (see text for details).

3.7. Results

3.7.1. Factors affecting tubule size of the pelvic and ventral glands

Tubule size may be determined by the time elapsed since a courtship encounter, but the number of spermatophores deposited during an encounter and the male's body size or reproductive condition may also contribute to overall tubule size.

Stepwise multiple regression was carried out to determine which parameters, such as male body size, crest height and time elapsed since mating were the best predictors of tubule size of the pelvic glands (Table 3.9) and the ventral glands (Table 3.10). The CSAs of the tubules, measured in the three positions of region C, were used to determine the multiple linear regression model. 'Position of the section' was one of the factors in the matrix to ensure that the slight changes in tubule size across region C did not mask any effects resulting from experimental manipulation.

Table 3.9. Stepwise multiple regression of parameters that may contribute to tubule size (CSA mm^2) of the pelvic glands (hours post deposition [time]; number of spermatophores deposited [spa]; snout-vent length [svl]; crest height [ch]; position of section in region C [section]). The table shows regression models after variables are removed from the equation, in turn, in order of least significance (n=8). Two sets of coefficients and p values are given: (i) taking into account all the parameters [initial equation], and (ii) after stepwise regression including only those variables which are significant ($p<0.05$) [final equation].

	initial equation $r^2=0.085$; F=42.20; p=0.00001		**final equation** $r^2=0.085$; F=52.73; p=0.00001	
parameters	*coefficient*	*p*	*coefficient*	*p*
Time	0.128	0.00001	0.124	0.00001
Section	0.141	0.00001	0.141	0.00001
Spa	0.182	0.00001	0.182	0.00001
svl	0.057	0.0179	0.058	0.015
ch	0.009	0.687		
intercept	0.005	0.162	0.005	0.161

Table 3.10. Stepwise multiple regression of parameters that may contribute to tubule size (CSA mm^2) in two fields of the ventral glands (n=8; hours post deposition [time]; number of spermatophores deposited [spa]; snout-vent length [svl]; crest height [ch]; position of section in region C [section]. The table shows regression models after variables are removed from the equation, in turn, in order of least significance. Two sets of coefficients and p values are given: (i) taking into account all the parameters [initial equation], and (ii) after stepwise regression including only those variables which are significant ($p<0.05$) [final equation].

FIELD A				
tubules adjacent to cloacal tube	**initial equation** r^2=0.094; F=22.31; p=0.00001		**final equation** (all the factors entered in the initial model were significant, therefore no further iterations were carried out)	
parameters	*coefficient*	*p*		
Time	-0.172	0.00001		
Section	-0.059	0.046		
Spa	-0.566	0.00001		
svl	-0.162	0.0005		
ch	0.623	0.00001		
intercept	2201.2	0.00001		
FIELD B				
tubules below the pseudopenis	**initial equation** r^2=0.140; F=30.76; p=0.00001		**final equation** r^2=0.139; F=50.88; p=0.00001	
parameters	*coefficient*	*p*	*coefficient*	*p*
Time	-0.202	0.00001	-0.219	0.00001
Section	0.0155	0.614		
Spa	-0.956	0.00001	-0.943	0.00001
svl	-0.043	0.318		
ch	0.935	0.00001	0.897	0.00001
intercept	4815.3	0.239	285.7	0.0005

The regression models that best describe the relationship between tubule size and various parameters that may contribute to tubule size, after stepwise multiple regression, are given in Table 3.11.

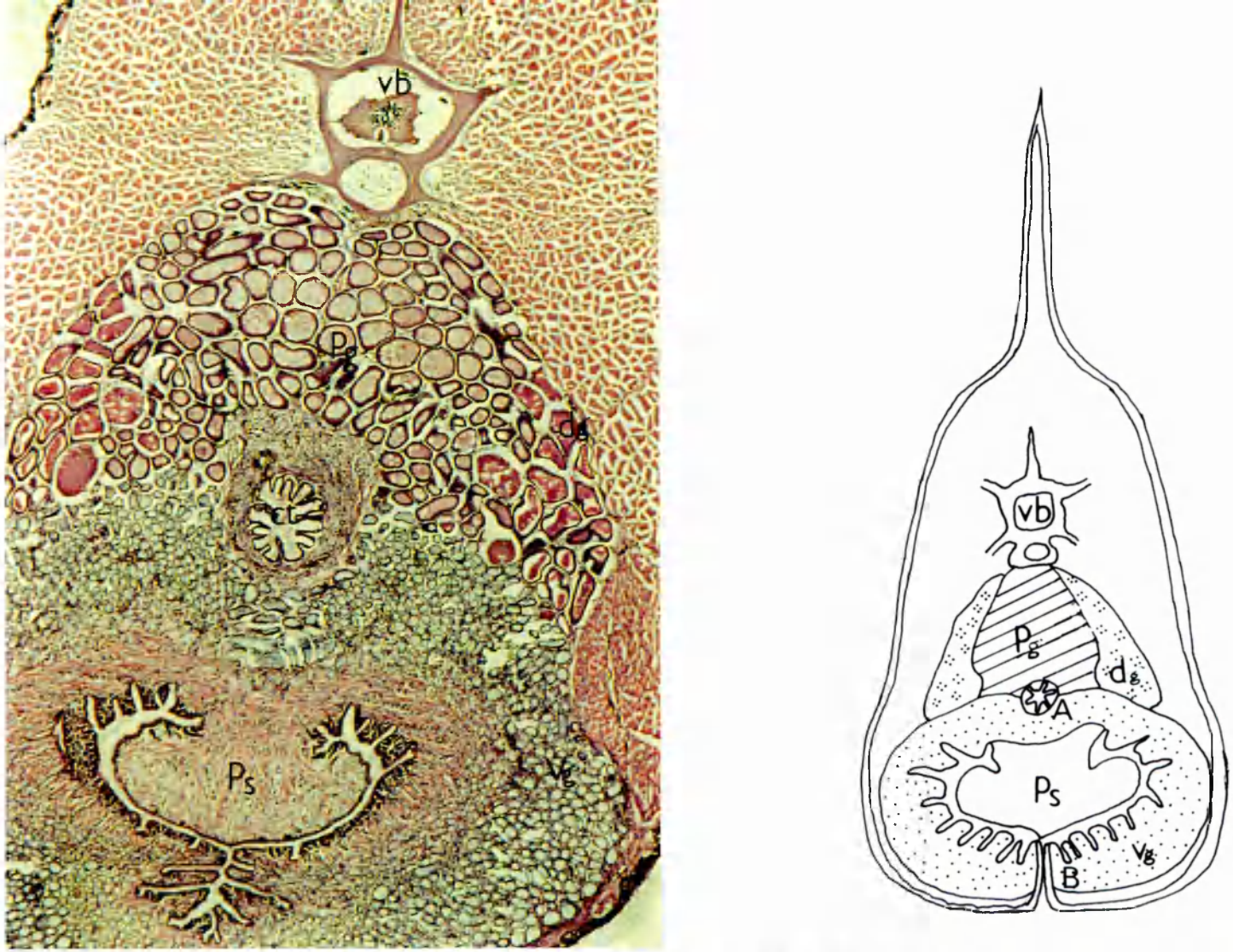


Fig 3.7a) A vertical section through the cloaca of the smooth newt in region C; b) drawing of vertical section through the cloaca in region C, showing position of the glands and the positions of the two fields [A and B] measured in the anterior ventral glands (see text for further details). Ps=pseudopenis; vb=vertebra; ct=cloacal tube; dg=dorsal glands; Pg=pelvic glands; Vg=ventral glands (anterior). Stained with haematoxylin and eosin (see text for details).

Fig 3.8 Vertical sections through a) tubules of the pelvic glands and b) tubules of the ventral glands (l=lumen, e=epithelial cells, ep=epidermis, Pg=pelvic glands, dg=dorsal glands; Vg= ventral glands; Ps=pseudopenis). Stained with haematoxylin and eosin (see text for details).

3.7. Results

3.7.1. Factors affecting tubule size of the pelvic and ventral glands

Tubule size may be determined by the time elapsed since a courtship encounter, but the number of spermatophores deposited during an encounter and the male's body size or reproductive condition may also contribute to overall tubule size.

Stepwise multiple regression was carried out to determine which parameters, such as male body size, crest height and time elapsed since mating were the best predictors of tubule size of the pelvic glands (Table 3.9) and the ventral glands (Table 3.10). The CSAs of the tubules, measured in the three positions of region C, were used to determine the multiple linear regression model. 'Position of the section' was one of the factors in the matrix to ensure that the slight changes in tubule size across region C did not mask any effects resulting from experimental manipulation.

Table 3.9. Stepwise multiple regression of parameters that may contribute to tubule size (CSA mm^2) of the pelvic glands (hours post deposition [time]; number of spermatophores deposited [spa]; snout-vent length [svl]; crest height [ch]; position of section in region C [section]). The table shows regression models after variables are removed from the equation, in turn, in order of least significance (n=8). Two sets of coefficients and p values are given: (i) taking into account all the parameters [initial equation], and (ii) after stepwise regression including only those variables which are significant ($p<0.05$) [final equation].

	initial equation $r^2=0.085$; F=42.20; p=0.00001		**final equation** $r^2=0.085$; F=52.73; p=0.00001	
parameters	*coefficient*	*p*	*coefficient*	*p*
Time	0.128	0.00001	0.124	0.00001
Section	0.141	0.00001	0.141	0.00001
Spa	0.182	0.00001	0.182	0.00001
svl	0.057	0.0179	0.058	0.015
ch	0.009	0.687		
intercept	0.005	0.162	0.005	0.161

Table 3.10. Stepwise multiple regression of parameters that may contribute to tubule size (CSA mm^2) in two fields of the ventral glands (n=8; hours post deposition [time]; number of spermatophores deposited [spa]; snout-vent length [svl]; crest height [ch]; position of section in region C [section]. The table shows regression models after variables are removed from the equation, in turn, in order of least significance. Two sets of coefficients and p values are given: (i) taking into account all the parameters [initial equation], and (ii) after stepwise regression including only those variables which are significant ($p<0.05$) [final equation].

FIELD A				
tubules adjacent to cloacal tube	**initial equation** $r^2=0.094$; F=22.31; p=0.00001		**final equation** (all the factors entered in the initial model were significant, therefore no further iterations were carried out)	
parameters	*coefficient*	*p*		
Time	-0.172	0.00001		
Section	-0.059	0.046		
Spa	-0.566	0.00001		
svl	-0.162	0.0005		
ch	0.623	0.00001		
intercept	2201.2	0.00001		
FIELD B				
tubules below the pseudopenis	**initial equation** $r^2=0.140$; F=30.76; p=0.00001		**final equation** $r^2=0.139$; F=50.88; p=0.00001	
parameters	*coefficient*	*p*	*coefficient*	*p*
Time	-0.202	0.00001	-0.219	0.00001
Section	0.0155	0.614		
Spa	-0.956	0.00001	-0.943	0.00001
svl	-0.043	0.318		
ch	0.935	0.00001	0.897	0.00001
intercept	4815.3	0.239	285.7	0.0005

The regression models that best describe the relationship between tubule size and various parameters that may contribute to tubule size, after stepwise multiple regression, are given in Table 3.11.

Table 3.11. Multiple linear regression models best describing the relationship between tubule size and various parameters that contribute to tubule size of the pelvic and ventral glands (cross-sectional area [CSA]; time post deposition [t]; number of spermatophores deposited [spa]; crest height [ch]; snout-vent length [svl]; position in region C of cloaca [f]).

Multiple linear regression model	**significance**
tubule size (CSA mm^2) of pelvic glands=0.005+0.124**t**+0.182**spa**+0.058**svl**+0.141**f**	p=0.00001
tubule size (CSA $micron^2$) of ventral glands, field A:=2201.2-0.172 **t**-0.566**spa**+0.623**ch**-0.162**svl**-0.059**f**	p=0.00001
tubule size (CSA $micron^2$) of ventral glands, field B:=285.7-0.219**t**-0.943**spa**+0.897**ch**	p=0.00001

As already shown, there is large variation in tubule size within the glands of an individual male. To determine whether all the tubules secrete the spermatophore base and matrix for the sperm mass or only those in the largest size classes, the CSAs of the 20 smallest and the 20 largest tubules in the pelvic glands were analysed separately. This analysis could not be carried out for the ventral glands because the sample size was too small. Stepwise multiple regression showed that the CSAs of the largest and the smallest tubules were related to the time elapsed since deposition (Table 3.12).

Table 3.12. Stepwise multiple regression of parameters that may contribute to tubule size (CSA mm^2) of a) the 20 smallest tubules and b) the 20 largest tubules of the pelvic glands (hours post deposition [time]; number of spermatophores deposited [spa]; snout-vent length [svl]; crest height [ch]; position of section in region C [section]). The table shows regression models after variables are removed from the equation, in turn, in order of least significance (n=8). Two sets of coefficients and p values are given: (i) taking into account all the parameters [initial equation], and (ii) after stepwise regression including only those variables which are significant ($p<0.05$) [final equation].

20 smallest tubules

	initial equation $r^2=0.43$; F=70.84; p=0.00001		**final equation** (all the factors entered in the initial model were significant, therefore no further iterations were carried out)	
parameters	*coefficient*	*p*		
Time	0.191	0.00001		
Section	0.448	0.00001		
Spa	0.204	0.00001		
svl	0.275	0.00001		
ch	0.967	0.012		
intercept	-0.775	0.00001		

20 largest tubules

	initial equation $r^2=0.31$; F=42.36; p=0.00001		**final equation** $r^2=0.31$; F=53.04; p=0.00001	
parameters	*coefficient*	*p*	*coefficient*	*p*
Time	0.400	0.00001	0.356	0.00001
Section	0.186	0.00001	0.186	0.00001
Spa	0.475	0.00001	0.476	0.00001
svl	-0.163	0.0005	-0.161	0.0004
ch	0.009	0.833		
intercept	0.044	0.00001	0.044	0.00001

3.8. Discussion

The various parameters that may affect tubule size of the cloacal glands were positively correlated with the cross-sectional areas (CSAs) of tubules in the pelvic glands and negatively correlated with CSAs of tubules in the ventral glands, with the exception of the relationship between crest height and tubule size. However, this difference may be an artefact of the methodology. The CSAs of tubules in the pelvic glands were measured from the outer edge of the secretory cells in the pelvic glands (across the secretory cells and the lumen) and from the inner edge in the ventral glands (across the lumen only). As the cells lining the lumen fill with secretory material again after courtship, the lumen may initially shrink in size until the material is released into the lumen, giving a negative correlation between tubule size and the various parameters. Thus the sign of the coefficients is not considered in the following discussions.

3.8.1. Is the number of spermatophores deposited during an encounter related to tubule size?

The number of spermatophores deposited during a courtship encounter (spermatophore score) was related to the CSAs of the tubules in the ventral and the pelvic glands (across all size classes and when the 20 largest or the 20 smallest tubules of the pelvic glands were considered separately). Thus males with cloacal glands containing larger tubules were able to deposit a higher number of spermatophores than males with cloacal glands containing smaller tubules, which is consistent with the rationale behind this study that larger tubules contain more secretory product than smaller tubules. Also, the positive relationship between tubule size and the time elapsed since deposition suggests that the tubule size increases concurrently with the amount of secretory product synthesised by the epithelial cells of a tubule. The relationship between tubule size and spermatophore score suggests that the amount of secretory product in the cloacal glands is one of the factors limiting spermatophore production during a single encounter in male *T. vulgaris*.

3.8.2. Is tubule size related to male body size or male reproductive condition?

In this study, the CSAs of tubules in the pelvic glands and the innermost ventral glands (Field A), but not of tubules in the outermost ventral glands (Field B) were related to male body size, measured as snout-vent length, whereas the CSAs of tubules in both fields of the ventral glands, but not of tubules in the pelvic glands, were related to crest height (an indicator of male reproductive condition). This difference between the parameters best predicting the CSAs of the tubules may be a consequence of the different positions of the tubules and glands within a male. Tubules of both the pelvic glands and the innermost ventral glands (field A) lie within the body of the newt, and thus are likely to be related to body size. However, tubules in the outer ventral glands (field B) lie outside the body of the newt, in the cloacal swelling, and the CSAs of these tubules may be less related to male body size and more related to reproductive condition (measured as crest height).

The swelling of the cloaca, which occurs in the breeding season, is due to hypertrophy of the ventral glands and may be under the same hormonal control as the development of the crest. In *Triturus carnifex*, testosterone and prolactin have been shown to act synergistically to control the development of the crest (Vellano *et al.* 1970) and to increase the turgidity of the cloacal swelling by acting on the connective tissue frame and the associated blood vessels (Mazzi & Biciotti 1976). Similarly, in *Cynops pyrrhogaster,* these hormones are involved in cloacal gland hypertrophy and secretion of the ventral glands, stimulating the production of mucopolysaccharides that constitute the spermatophore base (Kikuyama *et al.* 1975). The finding that crest height and tubule size in the ventral glands were positively correlated, despite measuring the internal CSAs of the tubules, suggests that tubule size in the ventral glands is more strongly correlated with crest height than with the other parameters, further supporting the hypothesis that the development of the crest and hypertrophy of the ventral glands are under the same hormonal control. Thus both the height of a male's crest and the size of tubules in the ventral glands may be determined by plasma testosterone levels, as

crest height has been shown to be correlated with the amount of glandular tissue, which synthesises testosterone, in the testes (Verrell *et al.* 1986).

These findings suggest that the tubules in the cloacal glands of larger newts and newts in better reproductive condition (measured as crest height) are bigger and contain more secretory product than the tubules in the cloacal glands of smaller newts, which is consistent with Verrell *et al.*'s study (1986) that showed that testes size in male newts is also related to male body size. By possessing larger cloacal glands and larger testes than smaller newts, larger males potentially are able to deposit more spermatophores than smaller newts, although the findings in the previous section have shown that larger males produce larger spermatophore bases, which may utilise more secretory product than the bases produced by smaller males. Similarly, males in good reproductive condition, which also are likely to be larger males as crest height shows positive allometry with male body size (Baker 1990a; Green 1991), may be able to deposit more spermatophores than males in poor condition because they have larger cloacal glands, but these relationships have not been observed during single courtship encounters (Halliday 1975; Baker 1990a; this study). Thus the relationship between spermatophore production and male body size will be investigated further, throughout a single breeding season, in chapter 5. Taken together, these studies suggest that larger males possess the ability to deposit more spermatophores during an encounter, but that other factors, such as body condition or time since last mating may determine whether this actually occurs.

3.8.3. Is the recovery period in male smooth newts correlated with replenishment of the secretory products of the cloacal glands?

Tubule size of both the pelvic and the ventral glands is predicted by the number of spermatophores deposited during a courtship encounter and the time elapsed after termination of the encounter. These two factors are expected to be linked if the physiological mechanisms producing the secretory materials are rate limiting, i.e. the time taken for the tubules to refill is related to the level of depletion. This study suggests that the number of

spermatophores deposited during an encounter is dependent on the amount of secretory products present in the glands. Males that have replenished the secretory products that were used in a previous encounter are able to deposit more spermatophores than males with only partially replenished glands. Although there was much variation between the CSAs of the tubules in each gland, the multiple regression model for the pelvic glands still held when the 20 largest tubules or the 20 smallest tubules were considered separately, suggesting that all the size classes of tubules contribute to the matrix of the sperm mass, become depleted and are then replenished. The same may be true for the ventral glands, but the sample sizes for these glands were too small for a similar analysis to be undertaken. Replenishment of the secretory products may be affected by several factors in addition to time elapsed since last mating, for example, the nutritional status or body condition of the individual may affect the rate of replenishment. The relationship between body condition and spermatophore production is discussed further in chapter 5.

If secretion of the spermatophore by the cloacal glands in smooth newts is under similar hormonal control to the production of pheromone by the dorsal gland in *Cynops pyrrhogaster* (Yamamoto *et al.* 1996), the rate of replenishment of the secretory products may show a concurrent decline with the levels of circulating testosterone as the season progresses.

These findings support the hypothesis that the behavioural recovery period, observed in male newts (Verrell 1986a) and salamanders (Sever & Houck 1985; Verrell 1988) after maximal spermatophore deposition, is a consequence of depletion of the secretory products of the cloacal glands. This finding demonstrates that it is the production of the sperm accessory materials, not the production of sperm *per se*, which limits male reproductive potential, and supports Dewsbury's (1982) hypothesis that ejaculate production may be costly.

In other species that transfer sperm via spermatophores, males require a recovery period after mating before they are able to produce a similar-sized spermatophore (Rutowski 1979; Oberhauser 1988; Simmons *et al.* 1992; Simmons 1995). Indeed, in some species males

delay remating until the spermatophore is similar in size to the first (Simmons 1990). In those species which deposit a large first spermatophore and smaller second and subsequent spermatophores, the size of the second spermatophore is also positively related to time elapsed since mating, e.g. in the butterflies *Papilio machaon* (Svärd & Wiklund 1986), and *Pieris napi* (Wiklund & Kaitala 1995). These studies suggest that the recovery period exhibited by the males is related to the replenishment of the sperm accessory materials. Indirect evidence has supported this hypothesis. For example, in an unnamed tettigoniid, Simmons (1990) found that males were 16% lighter after mating and subsequently increased in weight. Five days after mating, the male tettigoniids had regained their premating weight and begun calling again, suggesting that the increase in weight was a consequence of replenishment of the sperm accessory materials, the spermatophylax. My study provides direct evidence that the recovery period in smooth newts is related to replenishment of the secretory products of the cloacal glands, which secrete the spermatophore base and the matrix for the sperm mass.

3.8.4. Is spermatophore production constrained by cloacal gland capacity?

The findings in the two sections of this chapter suggest that spermatophore production is constrained by cloacal gland capacity. Tubule size limits the number of spermatophores deposited during a single courtship encounter, but utilisation of the secretory products within the tubules does not reduce the heights of the spermatophore bases (section 3.3). After maximal deposition of spermatophores, male newts exhibit a behavioural period of reduced mating ability, which is correlated with replenishment of the cloacal glands.

Several other factors, which may also influence the number of spermatophores that a male can deposit during a single encounter, may be worth investigating in future studies. The total number of tubules in the pelvic and ventral glands of each male was not determined in this study because it was not possible to tell whether an oblique cut was through one or several tubules. Computer reconstruction may enable the individual tubules to be followed throughout the cloaca and thus establish the definitive number of tubules present in each gland, although this is proving difficult to achieve in other salamanders (Sever pers comm).

Comparison of tubule number would determine whether males possessing more tubules in their glands are able to deposit more spermatophores than males possessing fewer tubules.

As mentioned previously, measuring the CSAs of tubules does not determine whether the size of each tubule reflects the absolute amount of secretory product contained in the lumen. By measuring the intensity of the stained material in the lumen of the tubules, the amount of secretory product may be quantified, which may reveal whether all the size classes of tubules fill to the same density. Measuring the amount of secretory product in the glands may also enable researchers to establish whether larger males replenish the secretory products faster than smaller males. If this scenario occurred, larger males would have a shorter refractory period than smaller males, enabling them to be ready to remate faster and potentially to achieve higher reproductive success, which could be confirmed by testing males, using standardised courtship encounter trials, at intervals up to 24 h after mating.

The outcome of all the predictions investigated in this study is summarised below (Table 3.13).

3.9. Summary

Spermatophore base height is correlated with male body size, giving further support to the hypothesis that spermatophore bases are moulded in the cloacal cavity.

The height of each spermatophore base is maintained during a single encounter and throughout the season, which may be a consequence of the morphology of the cloaca.

The size of the tubules of the cloacal glands is positively related to the time elapsed since termination of courtship. This study provides direct evidence that the behavioural period of reduced mating ability, termed the 'recovery period', observed after maximal deposition of spermatophores during a courtship encounter, is correlated with replenishment of the secretory products of the cloacal glands.

Male newts are constrained in their ability to produce spermatophores due to the physiological mechanisms controlling spermatophore production. The effect of these

constraints on male gametic strategies and mating success throughout the season is investigated in Chapters 4 and 5 respectively.

Table 3.13 Summary of hypotheses and predictions investigated in this study (Y=prediction supported; N=prediction not supported).

Hypothesis	**prediction**	
Hypothesis H_1 The spermatophore is moulded in the cloacal lumen.	*Prediction: 1* Spermatophore base height is determined by male body size.	**Y**
Hypothesis H_2 Successful pick-up of sperm masses by females is related to the height of the spermatophore base.	*Prediction: 2* Spermatophore base height is independent of male body size and is maintained across an encounter and throughout the season.	**N**
	Prediction: 3 Spermatophore base height is related to male tail depth.	**N**
Hypothesis H_3 Spermatophore production is constrained by cloacal gland capacity.	*Prediction: 4* Height of spermatophore base decreases; a) in subsequent sequences during an encounter, b) throughout the season.	**N**
	Prediction: 5 Height of spermatophore base is maintained a) in subsequent sequences during an encounter, b) throughout the season, but the number of spermatophores produced is limited.	**Y**
	Prediction: 6 The behavioural recovery period is a consequence of the requirement to replenish the secretory products of the pelvic and ventral glands.	**Y**

Chapter 4. Male gametic strategies: allocation of sperm to sperm masses of individual spermatophores

4.1. Introduction

Pitnick & Markow (1994) define the gametic strategy of a male in terms of the amount of energy invested in each gamete, the total energy invested in gamete production, and the pattern of allocation among successive ejaculates or mates. While acknowledging that these three traits have co-evolved, they advocate considering each trait as a discrete component. This study is concerned with one of these factors, namely, the pattern of allocation of sperm to sperm masses in successive spermatophores, deposited both in response to one female during a single encounter and in response to several females during one breeding season.

Although gametic strategies in females have been the subject of extensive study (Stearns 1992), the pattern of allocation of male reproductive effort has received little attention (Birkhead & Hunter 1990; Levitan 1993; Pitnick & Markow 1994). Allocation of sperm has evoked little interest, partly because, as emphasised in chapter 1, sperm have been considered to be cheap to produce and, therefore, the gametic strategy of males was thought to be an unvarying 'maximal insemination of as many females as possible' (Parker 1970).

Males exhibiting a maximal insemination strategy allocate large numbers of sperm to each ejaculate or spermatophore (c in Fig. 4.1), which may result in more sperm being transferred to each female than is needed to fertilise the full clutch of eggs (Bedford 1970; Brillard & Bakst 1991; Eady 1995) or that can be stored in the female reproductive tract (Hardy & Dent 1986a; Eady 1994). The transfer of 'excess' sperm may be a consequence of sperm

production (Cohen 1973) or may be an adaptive strategy, for example, the extra sperm may consist of different morphs that have functions other than fertilisation of the ova. The presence of non-fertilising morphs retards sexual receptivity in butterflies (Gage 1994) and prevents fertilising morphs belonging to a rival male from reaching the spermatheca in *Drosophila* (Snook *et al.* 1994) or reaching the site of fertilisation in humans (Baker & Bellis 1988; 1989; 1995, although this is disputed by Harcourt 1989; 1991). However, to date there is no evidence to suggest that different morphs are present in newt sperm.

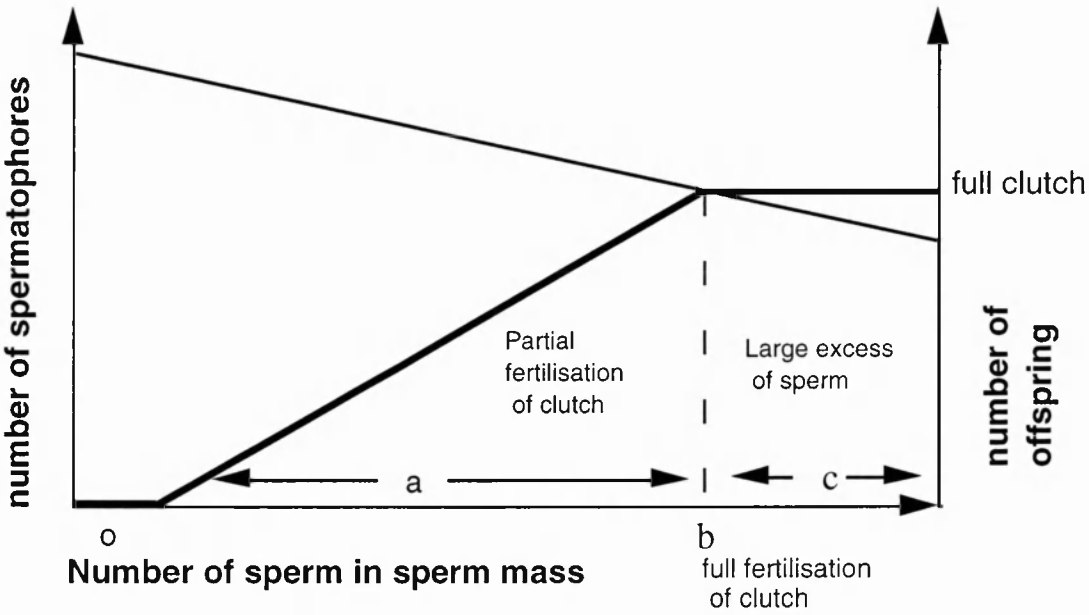


Fig 4.1. Model of allocation of sperm and spermatophores when the total amount of sperm available for use is limited [thick line=relationship between number of sperm in sperm mass and number of offspring produced, thin line=relationship between the number of spermatophores produced and the number of sperm in the sperm mass] a) represents the sperm conservation strategy of adjusting sperm per sperm mass according to the sociosexual environment, which may lead to partial fertilisation of the clutch of each female that the male inseminates. c) represents the maximal insemination strategy of spermatophores each containing a large number of sperm, but there is no concomitant increase in offspring, therefore, high costs may be incurred due to reduction in the number of sperm available for fertilisation of clutches of other females. b) represents optimisation of trade-off between number of sperm in a sperm mass and the number of spermatophores deposited so that the full clutch of each female inseminated by the male is fertilised (bet-hedging strategy), assuming that the female only mates with one male. o) too few sperm in a sperm mass for fertilisation to occur due to loss of sperm in the reproductive tract.

Insemination of 'excess' sperm may be one adaptive strategy which resolves opposing selection forces on paternity, namely, preemption of sperm competition and reducing the likelihood of the male's own sperm being superseded (Parker 1984). Alternatively, transfer of 'excess' sperm may ensure that the spermatheca is full of viable sperm. Sperm have to

negotiate the female tract to reach the spermatheca, and in many species female factors, such as low pH, phagocytosis and absorption by the tract wall may reduce the number of sperm entering the sperm storage organs (Birkhead *et al.* 1993). In some species these female factors are thought to enable selection of specific sperm into the storage organs, but evidence supporting this hypothesis is scarce (Eberhard 1996, also discussed further in chapter 6).

The theoretical models upon which the maximal insemination hypothesis is based, and the subsequent change in emphasis from production of individual sperm to production of ejaculates or spermatophores, have been discussed previously in Chapter 3. The major consequence of this change in emphasis has been the realisation that male reproductive success may be limited by physiological factors constraining the production of sperm accessory materials or the simultaneous production of the large numbers of sperm contained in each ejaculate or spermatophore. These physiological constraints of spermatophore or ejaculate production on male reproductive success may select for alternative male gametic strategies (Dewsbury 1982). Rather than always maximally inseminating each female, males may respond to the sociosexual environment and transfer differential amounts of sperm to the female depending on the situation (the sperm conservation hypothesis, a in Fig 4.1, Pitnick & Markow 1994).

The sperm conservation hypothesis is supported across several taxa, including insects (Svärd & Wiklund 1986; Gage & Barnard 1996), fish (Shapiro *et al.* 1994) and humans (Baker & Bellis 1993). In some species, males adjust their ejaculate in response to female body size (Baker & Bellis 1993; Shapiro *et al.* 1994; Gage & Barnard 1996); to virgin as opposed to previously mated females (Svärd & Wiklund 1986); or in response to the presence of rival males and the increased likelihood of sperm competition (Gage 1991; Gage & Baker 1991; Shapiro *et al.* 1994). As discussed previously, a male may gain a higher probability of fertilising ova by having a greater number of sperm in the female tract (Parker 1970; 1990). Thus, in the event of sperm competition, males transferring low numbers of sperm may be at a disadvantage. Shapiro *et al.* 1994) have shown in the bluehead wrasse *Thalassoma bifasciatum*, which exhibits both group and pair spawning,

that males in group spawns, despite having a smaller body size, release six times more sperm per ejaculate than males in pair spawns.

Furthermore, sub-maximal insemination, which results in the spermatheca being only partially filled after insemination by one male, occurs in some species, such as *Drosophila* (Gromko *et al.* 1984a; Pitnick & Markow 1994). In *Drosophila*, this strategy is thought to be related to female remating interval. If the interval before remating is sufficiently long to preclude sperm competition, selection favours males that transfer only sufficient sperm for utilisation by the female before she remates. Males of some species reduce the likelihood of sperm competition, and the requirement to inseminate females with high numbers of sperm, by transferring anti-attractants to the females along with the sperm, rendering the female less attractive to other males (Baumann 1974; Gromko *et al.* 1984a; Thornhill & Alcock 1983) or by producing a copulatory plug, which prevents insemination by a rival male (Thornhill & Alcock 1983). There is no evidence to suggest that male newts transfer anti-attractants to females, but in some species the base of the spermatophore or the matrix of the sperm mass may act as a temporary copulatory plug (Verrell 1991b; Rafinski pers comm).

If ejaculate size is determined before a mating bout commences, a male may be unable to vary the number of sperm contained within it. Thus an alternative strategy of sperm partitioning, termed bet-hedging, which does not require the male to be able to respond to the sociosexual environment, has also been proposed (Gillespie 1974; Slatkin 74; Seger & Brockmann 1987; Phillipie & Seger 1989). This strategy proposes that selection favours phenotypes with low variance in reproductive success over other phenotypes with higher variance, even though these phenotypes may have potentially higher mean fitness. In species with limited supplies of sperm, this hypothesis suggests that selection favours a strategy of partitioning gametes that reduces the variance in the number of sperm used by a male in each insemination (b in Fig 4.1). This strategy is more beneficial to males than the maximum insemination strategy, which would result in males, in such species, rapidly depleting their supplies of sperm after inseminating only a few females.

4.1.1. Gametic strategies in the male smooth newt

Smooth newts exhibit scramble competition polygyny, whereby males search for responsive mates and then compete for access to them (Verrell & McCabe 1988). Receptive females may be rare in the population, so a strategy of transferring large numbers of sperm, whenever a male successfully courts a responsive female, may ensure that he fathers most of their offspring. However, male newts arrive at a pond with a finite supply of sperm that is utilised during the current breeding season (Verrell *et al.* 1986). A previous study by Halliday (1976) has shown that males are able to deposit spermatophores over a large portion of the season, which may extend over several months. Similarly, females are known to mate, albeit infrequently, throughout the breeding season (Hosie 1992). Consequently, in order to maximise their reproductive success, males need to partition sperm into a series of spermatophores, both in the short term in response to a receptive female during a single encounter and in the longer term to enable them to deposit spermatophores to a series of females throughout the season. Males allocating large numbers of sperm to each sperm mass may run out of sperm before the season ends, thereby forfeiting some mating opportunities. Transfer of large numbers of sperm to each inseminated female may result in high numbers of offspring, which may compensate for any reduced mating opportunities and enable a male to maximise his reproductive success, but there are risks involved. A female may die before laying her full clutch, or she may remate before laying many eggs and the rest of her clutch may be fathered by the second male or she may fail to become inseminated and a large number of sperm may be wasted.

Thus gametic strategies in male smooth newts are constrained by the production and storage of sperm and sperm accessory materials and are influenced by sperm acquisition, storage and utilisation in the female. The combined effects of these selection pressures may result in males allocating sperm to each sperm mass according to one of the sperm partitioning strategies, rather than according to the maximal insemination strategy. From the literature, four potential opportunities for males to adjust the number of sperm per sperm mass in response to the sociosexual environment can be identified, namely,

according to the likelihood of successful pick-up of the sperm mass by the female's cloaca, according to the female's remating interval, in response to female body size (investigated in a separate study and found to be unsupported, Halliday, Waights & Hosie in prep) and in response to the presence of a rival male (beyond the scope of this study). The first two factors are investigated in this study and are discussed in more detail below.

Differential pick-up success

During staged pairings in a laboratory, a receptive female may elicit up to six spermatophores from the male and take up to four sperm masses into her cloaca (see references in chapter 1). Although in the wild males rarely deposit more than three spermatophores during an encounter and females seldom pick up more than one sperm mass (Kauffmann pers comm), multiple insemination does occur. Approximately 20% of the females in Kauffmann's study that did pick up sperm masses in their cloaca were doubly inseminated. Multiple insemination is considered to be a male strategy to preempt sperm competition, which utilises a fixed morphological and physiological regime for ejaculate production and the behavioural mechanism of multiple insemination to increase sperm numbers when sperm competition is likely to arise (Lanier *et al.* 1979; Parker 1984). Multiple insemination increases the number of sperm in the reproductive tract of the female (which is similar to the sperm loading hypothesis of Dickinson 1986), but allows the male several 'decision points' for sperm transfer, rather than only one as in the maximum insemination strategy. This hypothesis is supported in species that transfer sperm directly from the male to the female during copulation, e.g. in gerbils *Meriones unguiculatus* (Ågren 1990).

In species, such as smooth newts, that transfer sperm indirectly via a spermatophore deposited on the substrate, multiple deposition during an encounter may be a male strategy that increases the number of sperm in the female tract or may be a consequence of males seeking to ensure that the females are inseminated. Multiple deposition may lead to multiple insemination of the female, either because the male cannot tell if the last sperm mass was picked up, for example (if female receptivity does not change after pick-up) or

because the female actively seeks multiple insemination. Previous work has shown that females may preferentially pick up sperm masses from spermatophores deposited in particular ordinal positions. For example, Halliday (1974) has shown that female smooth newts are more likely to pick up the second and third spermatophores deposited during an encounter than the first. Other workers have found the reverse trend (for three spermatophores, Hosie 1992) or no trend (for two spermatophores, Hosie 1992; Kauffmann pers comm; this study), but these conflicting findings may simply reflect differences in the temperature at which each study was conducted. Males courting at higher temperatures may proceed to spermatophore deposition before the female is correctly oriented for successful pick-up of the sperm mass. If pick-up success of sperm masses in particular ordinal positions is determined by a female's intrinsic motivational level, a male may be able to respond to this and allocate differential numbers of sperm to each sperm mass, in accordance with its likelihood of being successfully transferred to the female.

Even if insemination does occur during an encounter, males are not assured of fertilisation success. Sperm are stored in the females' spermathecae and fertilisation does not occur until just prior to oviposition of each individual egg, which may be days (Diaz-Paniagua 1989; Hosie 1992; this study) or weeks later (Bell & Lawton 1975; Verrell & Halliday 1985; Verrell & McCabe 1988). Male newts do not guard females until oviposition occurs, so females are able to remate during this period without their first partners being aware or being able to react. The effect of multiple mating by females on male gametic strategies is discussed in the next section.

Female's remating interval

The peak of sexual activity, in a given population, occurs at the beginning of the breeding season (Verrell & Halliday 1985; Verrell & McCabe 1988), prior to the synchronous onset of oviposition (Verrell & McCabe 1988), when female newts are highly receptive and mate multiply (Hosie 1992). The operational sex ratio at this time is least male-biased and may even be female-biased (Verrell & McCabe 1988), which may lead to selection favouring

males that allocate sperm according to the female's remating interval so that they are ensured fertilisation success, albeit of a portion of the female's clutch. However, if females remate prior to oviposition, sperm competition theory predicts that males should only allocate sperm according to the female's remating interval when there is high last-male precedence, i.e. virtually complete displacement of the first male's sperm. Complete displacement is more likely to occur in species, such as smooth newts, that store sperm in blind-ended spermathecae in which sperm layering can occur (Walker 1980), although Ridley (1989) found that last-male precedence was more highly correlated with the natural remating frequency of females.

Sperm competition has only been studied in two species of urodeles. The study in the salamander *Desmognathus ochrophaeus* excluded last-male precedence, as none of the clutches were sired totally by the last male to mate (last male paternity ranged from 5-69% Houck *et al.* 1985a). However, plethodontids possess a compound spermatheca in which the blind-ended tubules are joined together by a common neck (Sever 1994a), which may influence sperm competition, independently of mating order.

A study of *Triturus alpestris* (Rafinski pers comm) has demonstrated last-male precedence, but not in all pairings. In some females, double mating resulted in perfect mixing and even first-male precedence (Rafinski pers comm). In double matings occurring prior to ovulation or as oviposition commenced, second-male precedence was dependent on the time interval between the two matings, ranging from 0.6% when the matings were only a few minutes apart to 100% when the interval was ten days, supporting Ridley's (1989) findings and suggesting that the sperm mass may act, temporarily, as a copulatory plug. Females reinseminated by a second male during their oviposition period subsequently laid eggs fathered by the second male. After a period of egg-laying, some females reverted back to laying eggs fathered by the first male. This result gives good support for the sperm stratification hypothesis (Lessels & Birkhead 1990), but is surprising in the light of possible deterioration and phagocytosis of sperm within the spermathecae, which is known to occur in other urodeles (Sever 1991; Sever & Brunette 1993; Brizzi *et al.* 1995b). One possible

explanation is that the first male transfers more sperm to the female than the second male. Therefore, the sperm from the second male is depleted more quickly, due to production of offspring and maybe phagocytosis of the sperm, than the sperm from the first male. The possibility should also be considered that this result is a consequence of intrinsic male or female factors rather than mating order. It has been shown that males of different genotypes often exhibit differential fertilising capacity, due to differential sperm mobility, time to capacitation, and ability to penetrate the ovum, e.g. in rats (Lanier *et al.* 1979; Martin & Dzuik 1977; Dewsbury & Sawrey 1984) and in golden hamsters (Huck *et al.* 1985). Males also achieve differential paternity dependent on the female. In *Desmognathus ochrophaeus* some females are more successful at eliciting and picking up spermatophores (Houck *et al.* 1985b; Shillington & Verrell 1996), but whether these intrinsic factors affecting mating success result in differential paternity of offspring remains to be investigated.

In contrast to the beginning of the season, females remate at irregular intervals during the oviposition period, which causes the operational sex ratio to become very male-biased. Males are subjected to strong intrasexual competition, with a low probability of acquiring multiple mates, which could lead to selection favouring the transfer of large numbers of sperm in each sperm mass, to ensure fertilisation of the remaining clutch. Synchronous onset of ovulation and the subsequent decline in female remating rate suggests that males may need to be able to switch sperm allocation strategies according to whether most females in the population are highly receptive and mating multiply (pre-ovulatory) or are unreceptive and mating only occasionally (post-ovulatory). Within a population, arrival at the pond is not synchronous for either sex (Verrell & Halliday 1985), so individual males may exhibit different strategies, allocating sperm according to the social environment at the time of each mating.

4.1.2 Physiological constraints on the allocation of sperm

As discussed previously, sperm allocation in smooth newts is limited by a finite supply of sperm, but males within a population may not all be constrained to the same degree. Males

exhibit a large range in body size, which may lead to differential seasonal supplies of sperm because testes size in smooth newts (Verrell *et al.* 1986), in common with many other species (Møller 1988a; 1988b; 1989), is positively correlated with body size. Testes size is also positively correlated with the number of sperm contained in an ejaculate, both within species, e.g. in human males (Baker & Bellis 1995) and across species, e.g. in primates (Møller 1988a) and in butterflies (Gage 1994), which suggests that the number of sperm contained in the sperm masses of male smooth newts may also be related to male body size. Similarly, the proportion of the testes that comprise glandular tissue, which may be indicative of the release of higher numbers of sperm into the vasa deferentia (discussed further in chapter 5), is higher in males in better reproductive condition (measured as crest height) than in males in poorer condition. Thus larger males and males in better reproductive condition are predicted to deposit similar or higher numbers of spermatophores, each containing higher numbers of sperm, than smaller males or males in poorer reproductive condition (Verrell *et al.* 1986).

Male newts are also constrained by their production of sperm accessory materials, which may be influenced by body size (chapter 3), stored reserves, or by current energy intake. Therefore, individual males within a population may be exhibiting different strategies according to their ability to produce spermatophores, their supply of sperm and the local sociosexual environment. Individual males may also respond to the decreasing availability of sperm accessory materials, during an encounter or across the season, by decreasing the numbers of sperm allocated to the sperm masses of successive spermatophores, irrespective of the strategy exhibited.

4.2. Aims

This study investigates the pattern of sperm allocation to successive sperm masses deposited during single courtship encounters by a series of males, each tested once only, throughout a breeding season.

4.2.1. Hypotheses and predictions

The data were used to test the hypotheses outlined in Table 4.1.

Table 4.1 Hypotheses for the allocation of sperm in male smooth newts.

Gametic Strategies
Hypothesis: H_1 Males allocate sperm to each sperm mass according to the maximal insemination hypothesis.
prediction: 1 Each sperm mass will contain sufficient sperm to fertilise a female's full clutch.
Hypothesis: H_2 Males allocate sperm to each sperm mass according to the current sociosexual environment (the sperm conservation hypothesis).
prediction: 2 Males allocate sperm to each sperm mass according to the likelihood that it will be picked up by the female's cloaca, e.g. allocating more sperm to sperm masses deposited later (Halliday 1974) or earlier in the encounter (Hosie 1992).
prediction: 3 Males allocate sperm to each sperm mass according to the female's remating interval, e.g. allocating more sperm to sperm masses deposited during oviposition. At this time, each female mates infrequently (Verrell & McCabe 1988; Hosie 1992) and the last male to mate gains the precedence in sperm competition and fathers a higher proportion of her offspring.
prediction: 4 Males allocate sperm to each sperm mass according to female body size, i.e. allocating more sperm to sperm masses deposited in response to larger females (shown not to occur in a separate study, Halliday, Waights & Hosie in prep).
prediction: 5 Males allocate more sperm to sperm masses deposited in the presence of a rival (not tested in this study).
Physiological constraints on sperm allocation: differential sperm supply
Hypothesis: H_3 Allocation of sperm per sperm mass is related to male body size or male reproductive condition.
prediction: 6 Larger males or males in better reproductive condition will allocate more sperm per sperm mass than smaller males or males in poorer reproductive condition.
Physiological constraints on sperm allocation: production of sperm accessory materials
Hypothesis: H_4 Allocation of sperm per sperm mass is limited by the production of sperm accessory materials and the finite supply of sperm.
prediction: 7 During an encounter the number of sperm contained in successive sperm masses will decrease as the amount of matrix available for binding the sperm decreases.
prediction: 8 The number of spermatophores deposited during each encounter and the sperm content per sperm mass will decrease as the season progresses.

4.3. Methods

In smooth newts, the sperm contained within the sperm mass are tightly coiled together, making it difficult to determine the number of sperm present in the sperm mass using conventional methods of dilution and counting in a haemocytometer. Consequently, an indirect method was developed in which the amount of DNA in the sperm mass was determined.

4.3.1. Experimental protocol

The following methods have been described previously:- collection and maintenance of newts (section 3.3.1), production and collection of spermatophores (section 3.3.2).

4.3.2. Determination of the amount of DNA in sperm masses

DNA content of the sperm mass was determined by a fluorescent assay adapted from McAmble (Conn's laboratory pers comm), which in turn was based on a method developed by Labarca & Paigen (1980). DNA content in crude tissue homogenates is determined by the enhancement of fluorescence when bis-benzamide (Hoechst 33258) binds to DNA. DNA is rendered accessible to the fluorochrome by dissociating the chromatin with a high salt buffer. The reaction between DNA and Hoechst 33258 occurs on mixing and is stable for 16 h (in the dark). The reaction is linear with increasing concentrations of DNA in the range 0.2-5.0x10^{-6} g ml^{-1}, so is suitable as a sensitive assay technique. The method is described below and outlined in Table 4.2.

4.3.3. Tissue preparation

The vial containing the sperm mass was allowed to thaw and then refrozen to disrupt the cell membranes and aid dissociation of DNA. The sperm mass was homogenised in a glass homogeniser for 1 minute and the homogenate was centrifuged to remove the cell debris. The resulting supernatant was frozen until assayed. To check that all the released DNA was present in the supernatant, eight pellets were resuspended in water, centrifuged and the

second supernatant assayed. This second supernatant gave values that were no different to the values for the blank.

Table 4.2. The method for analysing the amount of DNA in the sperm mass of a spermatophore is outlined below (See text for details).

DNA FLUORIMETRIC ASSAY (adapted from Craig McAmble, Conn's Laboratory 1985)
Suspend sperm mass in 1 ml distilled water.
Freeze and thaw to disrupt cell membranes.
Break up sperm mass using ground glass homogeniser.
Centrifuge homogenate: 20°C, 3 100 rpm, 10 min.
Pipette off supernatant and store frozen (-18°C) until assayed.
Standard Curve produced for each assay, 0.2 - 5.0x10^{-6} g Calf Thymus DNA ml^{-1} against fluorescence.
DNA content per sperm mass calculated.

4.3.4. Assay

Method

The Hoescht 33258 and single stranded DNA (calf thymus) were purchased from Sigma. The double strength assay solution consisted of 0.1 M $NaPO_4$, 4 M NaCl, pH 7.4, containing 2x10^{-6} g ml^{-1} Hoescht 33258. A series of standard concentrations of DNA (0.2-5.0 x10^{-6} g ml^{-1}) were made up in distilled water (see Fig 4.2). One ml of assay solution was added to one ml of standard or sample giving a final concentration in the tube [0.05 M $NaPO_4$, 2 M NaCl, pH 7.4, containing 1x10^{-6} g ml^{-1} Hoescht 33258] which corresponded to the assay solution used by Labarca & Paigen (1980). A standard curve for concentration of DNA in the range 0.2-5.0x10^{-6} g ml^{-1} against fluorescence emitted at 492 nm was used to determine the concentration of DNA of the samples (Fig 4.2). The fluorescence was determined using an excitation wavelength of 356 nm in a Perkin-Elmer fluorescence spectrophotometer. Duplicate tubes were set up for both standards and samples, including a reagent blank [1 ml assay solution, 1 ml distilled water], whose value was deducted from the

value obtained for each standard or sample. Use of a reagent blank, ensuring that the line passes through the origin, facilitates comparison of the standard curves obtained for each assay run. The assay mixture was allow to equilibrate for 5 minutes before reading. After equilibration, the linearity of the standard curve and the duplicity of the standards and samples were very good. The mean of the readings for each sample was determined.

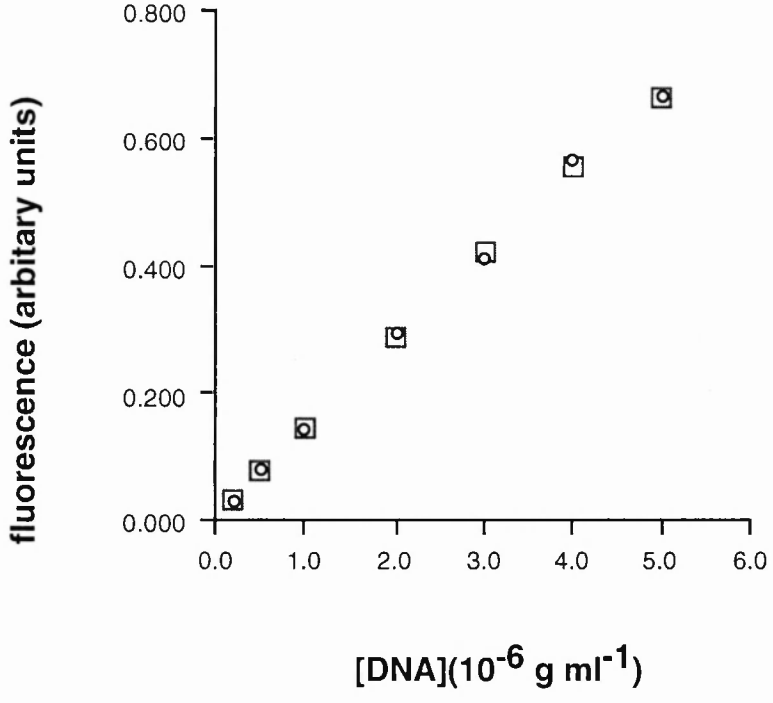


Fig. 4.2. An example of the standard curve for concentration of DNA against fluorescence (o=duplicate 1; □=duplicate 2). Excitation wavelength=356 nm, emission wavelength=492 nm.

4.3.5. Calculation of number of sperm per sperm mass

The number of sperm in a sperm mass is equal to the amount of DNA in the sperm mass divided by the amount of DNA contained in a single sperm. Each sperm contains the haploid complement of chromosomes (the 1C-value) for the species. The 1C-value for *Triturus vulgaris* has been determined by several workers (King 1990) to give a mean value of 23.6×10^{-12} g DNA.

The number of sperm per sperm mass = [concentration of DNA in the sperm mass] / [1c-value]
= [mean value from assay] / [1c-value for *T. vulgaris*]

For example, if the mean value from the assay = 2.5×10^{-6} g DNA

The number of sperm per sperm mass $= [2.5 \times 10^{-6}] / [2.36 \times 10^{-11}]$
$= 1.06 \times 10^{5}$ sperm
or 106,000 sperm per sperm mass

The accuracy of this method of determining the number of sperm in the sperm mass will be influenced by the presence of morphs with either macro, micro or multiple sperm heads and by aneuploidy (abnormal chromosome number) arising from the chromosomes failing to separate fully during spermatogenic cell division, but there is no evidence to date of either different morphs or aneuploidy occurring in newt sperm. To minimise any effects of differing amounts of DNA per individual sperm, the amount of DNA in individual sperm masses was used directly for comparative purposes, rather than estimating sperm number.

4.4. Results

4.4.1. Variation in the number of spermatophores deposited during an encounter

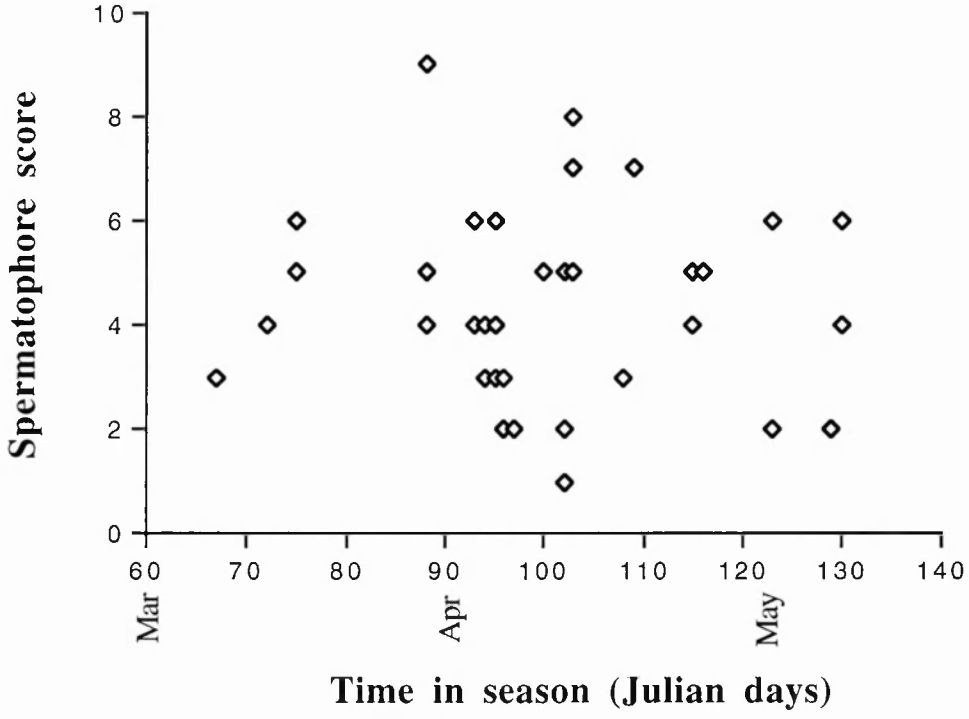
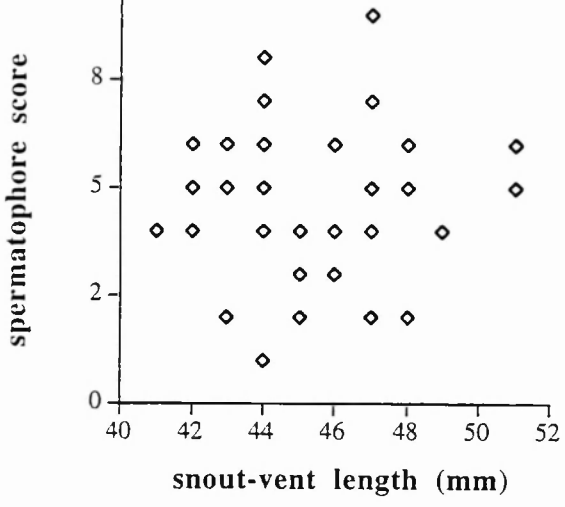
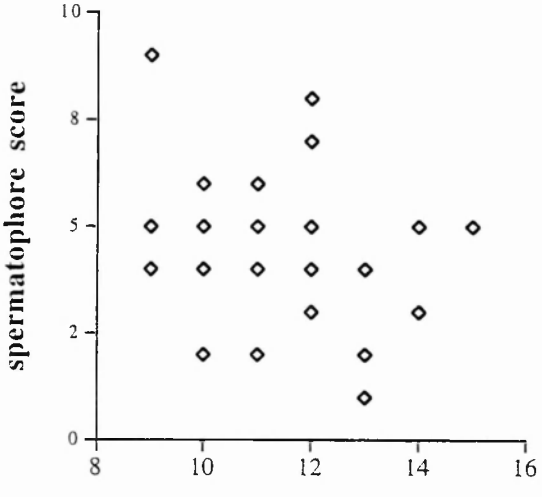


Fig 4.3. Variation in the total number of spermatophores [spermatophore score] deposited by individual males during single courtship encounters throughout the season (time). Each point represents an individual male tested once. (Partial pearson product moment correlation: n=47; df=43; $r_{spa\ time;svl\ td}$=0.28; total number of spermatophores deposited [spa]; time in season [time]; snout-vent length [svl]; tail depth [td]).

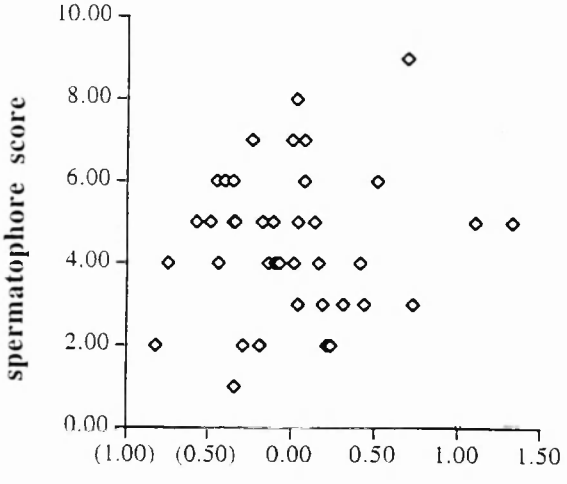
Throughout the season, some males tested were able to deposit only one or two spermatophores during a single encounter, whereas other males deposited six or more (Fig 4.3).



a)



b)



c)

Fig 4.4 Relationship between the total number of spermatophores deposited during a single courtship encounter [spermatophore score] and a) male body size, measured as snout-vent length ($r_{spa\ svl;\ td\ time}$=0.155; p>0.05), b) reproductive condition, measured as tail depth ($r_{spa\ td;\ svl\ time}$=0.27; p>0.05) and c) male body condition ($r_{spa\ bc;\ td\ time}$=0.41; p<0.05). (Pearson product moment partial correlations: n=41, df=37; total number of spermatophores deposited [spa]; time in season [time]; snout-vent length [svl]; tail depth [td]; body condition [bc]. The numbers in brackets are negative).

Pearson product moment partial correlations revealed that the total number of spermatophores deposited during a single encounter (spermatophore score) was not related to time in season, when the snout-vent length and tail-depth of the males were held constant (Fig 4.3). Spermatophore score was not related to male body size (measured as snout-vent length), or tail height, which may be an indicator of male reproductive condition, but was positively related to the body condition of the male, measured at the time of capture from the pond (Fig 4.4). Male body condition was determined by the residual method (Stearns 1992) which has already be used in this context (Baker 1990a) and is explained fully in chapter 5.

4.4.2. Variation in the number of sperm per sperm mass

The number of sperm per sperm mass in the sample of spermatophores analysed in this study ranged from 38 000 to 148 000 (Table 4.3).

Table 4.3. Range of amount of DNA and number of sperm in individual sperm masses (n=145).

Estimate per spermatophore	mean± s.d.	median	range
[DNA](g)	$1.68\pm0.40\times10^{-6}$	1.65×10^{-6}	$0.9\text{-}3.5\times10^{-6}$
Sperm number	$0.71\pm0.17\times10^{5}$	0.70×10^{5}	$0.38\text{-}1.48\times10^{5}$

4.4.3. Allocation of sperm to individual sperm masses during a single courtship encounter

The sperm masses deposited by individuals during a single courtship encounter were allocated to categories according to the ordinal position in which they were deposited (Fig 4.5). The amount of DNA in the second and subsequent sperm masses was compared to the amount of DNA in the first sperm mass, using repeated measures ANOVA (SPSS). In this method, the DNA content of each sperm mass of a particular category was compared with the DNA content of a corresponding sperm mass in the first category that had been deposited by the same individual, so each category did not need to contain the same number of spermatophores.

The amount of DNA in the second (n=24, $F_{(1,23)}$=0.57, p=0.46) and third (n=21, $F_{(1,20)}$ =0.14, p=0.71) sperm masses was not significantly different from the amount of DNA in the first sperm mass, but the amount of DNA in the fourth (n=16, $F_{(1,15)}$=4.59, p=0.05) and fifth (n=10, $F_{(1,9)}$=8.87, p=0.02) sperm masses was significantly reduced. The mean value of the DNA content of the sixth sperm mass (n=5, $F_{(1,4)}$=5.61, p=0.11) was also reduced compared with the first, although not significantly, which may be due to small sample size.

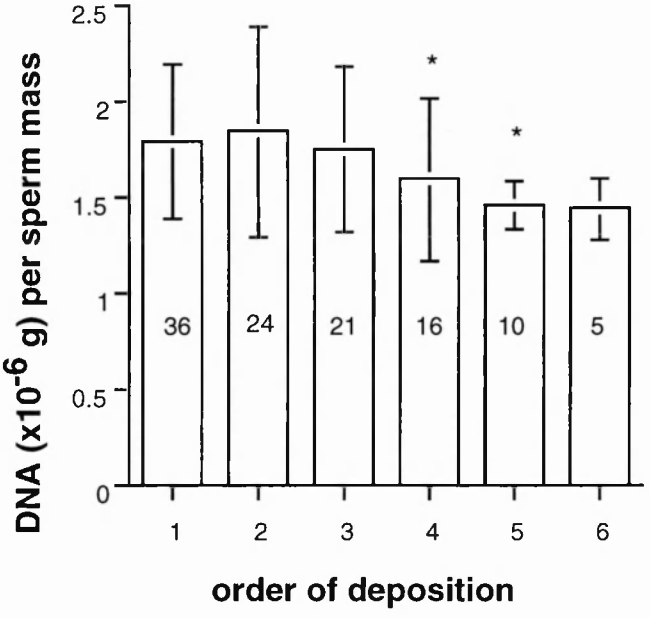


Fig 4.5. Amount of DNA in the sperm mass (mean±s.d.) of successive spermatophores, deposited during single courtship encounters. Numbers within the bars indicate the number of sperm masses in each deposition category. The asterisk denotes a significant difference between the amount of DNA in sperm masses in that category compared with the amount of DNA in the first sperm mass ($p<0.05$). The amount of DNA in the sperm masses was compared using repeated measures ANOVA (SPSS).

4.4.4. Variation in the number of sperm per sperm mass in relation to the total number of spermatophores deposited

From Fig 4.3. it can be seen that, throughout the season, some males only deposit two or three spermatophores during their test encounter while other males deposit five or six. This variation in spermatophore number may represent different male strategies, whereby males allocate high numbers of sperm to one or two spermatophores or divide the same amount of sperm among three or more spermatophores, depending on the availability of sperm accessory materials. To investigate whether the number of sperm contained in a sperm mass

is related to the total number of spermatophores deposited during the encounter (spermatophore score), the sperm masses were separated into categories according to the spermatophore score obtained. To ensure that any differences between the sperm numbers in the sperm masses were a result of different male gametic strategies prior to the onset of the encounter, rather than a consequence of depletion of sperm accessory materials or the number of available sperm during the encounter (see section 4.4.3 above), only the sperm masses from the first spermatophore deposited were analysed. Analysis by Pearson product moment partial correlation revealed that the DNA content of the first spermatophore deposited was not correlated with the total number of spermatophores deposited during a particular encounter (Fig 4.6), so males were not exhibiting different strategies.

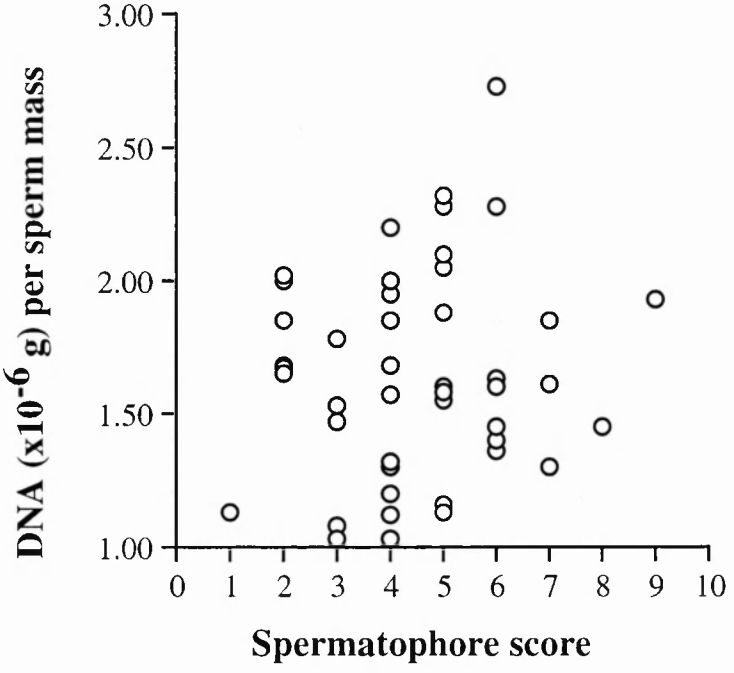


Fig 4.6 Comparison between the amount of DNA in the first spermatophore deposited and the total number of spermatophores deposited (spermatophore score). Partial Pearson product moment correlation: n=46; df=41; $r_{dna\ spa;\ td\ svl\ time}$= 0.115; p>0.05. (total number of spermatophores deposited [spa]; time in season [time]; snout-vent length [svl]; tail depth [td]).

4.4.5. Variation in the number of sperm per sperm mass in relation to body size, body condition and reproductive condition

To investigate the relationships between the number of sperm allocated to individual sperm masses and male body size, body condition and reproductive condition (estimated by tail

height), any confounding effects, resulting from males allocating differential numbers of sperm to successive spermatophores during an encounter, need to be eliminated. Therefore, only the amount of DNA in the sperm mass of the first spermatophore deposited by each male was used.

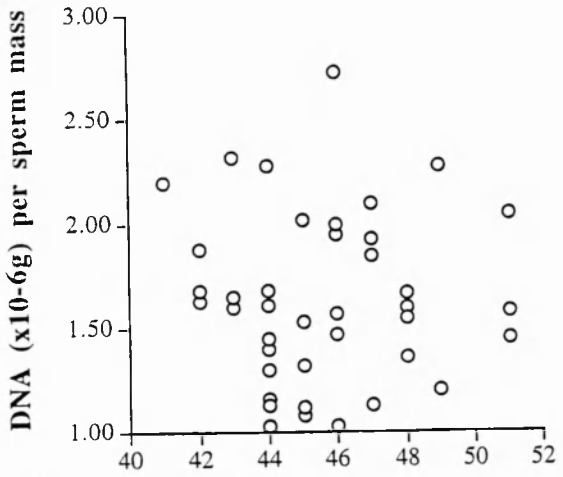
Table 4.4. Range of DNA content per spermatophore, for the first spermatophore deposited by each male, across the season (score=total number of spermatophores deposited by a male during a single encounter).

n	score	DNA range ($x10^{-6}$ g)	Sperm number range (1000)	DNA mean±s.d. ($x10^{-6}$ g)	DNA median ($x10^{-6}$ g)
46	2-8	1.03-2.73	44-116	1.66 ±0.39	1.62

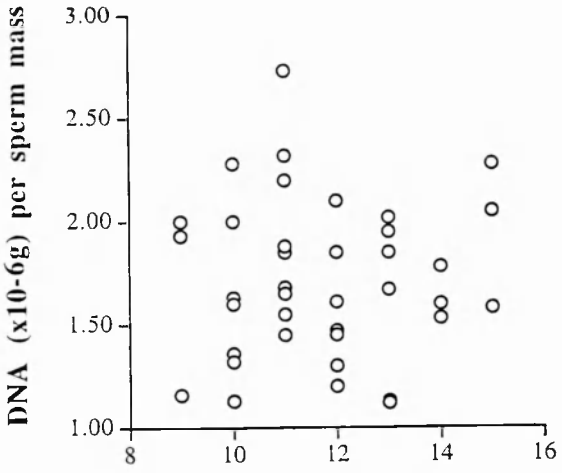
Despite an almost three-fold difference between the lowest and highest values (Table 4.4), partial correlations revealed that there was no relationship between the number of sperm in the sperm mass and male body size (measured as snout-vent length), or between sperm number and male reproductive condition (measured as tail height), or between sperm number and body condition (Fig 4.7).

4.4.6. Allocation of sperm to sperm masses during single courtship encounters across the breeding season

There was a significant increase in the amount of DNA per sperm mass across the season (Pearson product moment partial correlation: n=145; df=140; $r_{\text{dna time;svl td spa}}=0.21$; $p<0.05$) when all the spermatophores deposited throughout the season were considered, irrespective of their order of deposition within a single encounter. Since the amount of DNA in the sperm mass decreases with successive depositions and it was not possible to collect and analyse all the spermatophores deposited by each male, this finding may be confounded by the ordinal positions of the sperm masses contributing to the data set.



a.



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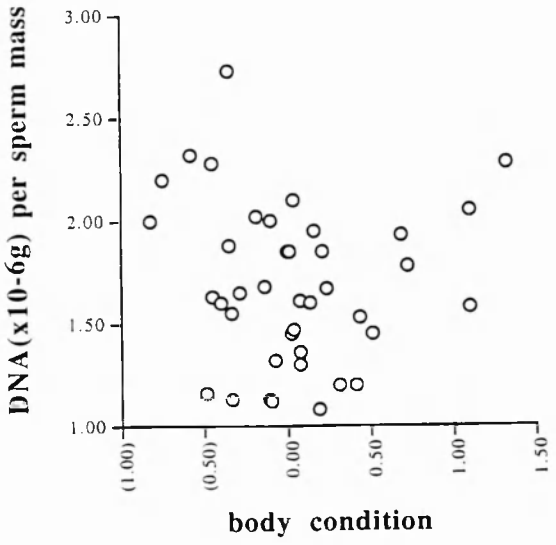


c.

Fig 4.7 Relationship between the amount of DNA in the first spermatophore deposited during a single courtship encounter and a) male snout-vent length ($r_{dna\ svl;\ spa\ td\ time}$=0.026; p>0.05), b) tail depth ($r_{dna\ td;\ spa\ svl\ time}$=0.01; p>0.05) and c) body condition ($r_{dna\ bc;spa\ td\ time}$=0.08; p>0.05). (Pearson product moment partial correlations: n=41; df=36; amount of DNA [dna]; total number of spermatophores deposited [spa]; time in season [time]; snout-vent length [svl]; tail depth [td]; body condition [bc]).

To investigate whether sperm masses deposited later in the season contained similar numbers of sperm compared with those deposited early in the season, the problem highlighted above was overcome by allocating sperm masses to categories dependent on the ordinal position in which they were deposited. The relationship between the amount of DNA in the sperm masses and time in season was determined for each deposition category (Table 4.5).

Table 4.5 Relationship between the amount of DNA in the sperm mass and time in season for the first five spermatophores deposited by individual males during a single courtship encounter (Pearson product moment partial correlations: *p<0.05)

ordinal position	n	df	partial correlation	p
first	46	41	$r_{dna\ time;spa\ svl,\ td} = 0.20$	>0.05
second	34	29	$r_{dna\ time;spa\ svl,\ td} = 0.07$	>0.05
third	34	29	$r_{dna\ time;spa\ svl,\ td} = 0.18$	>0.05
fourth	29	24	$r_{dna\ time;spa\ svl,\ td} = 0.53$	<0.05*
fifth	14	9	$r_{dna\ time;spa\ svl,\ td} = 0.23$	>0.05

The DNA content of the sperm masses of the first, second, third, and fifth spermatophores to be deposited during a courtship encounter did not increase as the season progressed, although the DNA content of the fourth sperm mass did increase significantly with time in season. The lack of relationship between the amount of DNA in the fifth sperm mass and time in season may be due to the small sample size.

4.5. Discussion

4.5.1. Do male newts allocate sperm in response to the sociosexual environment?

Male smooth newts might potentially respond to the sociosexual environment and adjust the number of sperm allocated to each sperm mass according to the likelihood of successful transfer to the female, the likelihood of the female remating, in response to female body size or in response to the presence of rival males. The prediction that the number of sperm

allocated to each sperm mass will reflect the likelihood of successful pick-up by the female, which has been shown to increase (Halliday 1974) or decrease (Hosie 1992) with order of deposition (over the first three spermatophores), was not supported in my study, as the sperm masses of the first three spermatophores deposited during an encounter contained similar numbers of sperm. The prediction that males are able to adjust the number of sperm allocated to sperm masses with respect to the likelihood of the female remating was weakly supported, in that the number of sperm in the fourth sperm mass deposited during an encounter increased across the season, as female remating rate declined (Verrell & McCabe 1988; Hosie 1992). However, for the sperm conservation hypothesis to be fully supported, one would expect all the sperm masses deposited during encounters towards the end of the breeding season to have elevated sperm numbers.

Another prediction of the sperm conservation hypothesis, that males allocate sperm to sperm masses in response to female body size, was investigated in a separate study and found to be unsupported (Halliday, Waights & Hosie in prep). Thus, taken together, these studies suggest that males are unable to vary the number of sperm allocated to each sperm mass in response to the sociosexual environment. This conclusion needs to be further examined because another prediction of the sperm conservation hypothesis, namely, that males respond to the sociosexual environment by allocating more sperm to sperm masses deposited in the presence of rival males, has not been investigated.

4.5.2. Does each sperm mass contain sufficient sperm to fertilise a full clutch of eggs?

The clutch size of female British *T. vulgaris* has been estimated as 100-600 eggs (Baker 1992a). This study estimated that each sperm mass contains between 38 000 and 148 000 sperm, so each sperm mass should be capable of fertilising a whole clutch of eggs. However, as discussed in chapter 1, it is important to determine whether the transfer of just one spermatophore can result in fertilisation of the full clutch as intrinsic female factors, such as sperm storage, polyspermy (in which more than one sperm enters each ovum) or the

females' pattern of egg-laying may limit male fertilisation success. It is known in many species that only a tiny fraction of the total number of sperm transferred to the female actually reach the site of fertilisation, e.g. in insects (Eady 1994), in birds (Birkhead *et al.* 1993) and in mammals (Suaraz *et al.* 1990), so a numerical determination of sperm number is not enough to demonstrate that sufficient sperm will be transferred to a female, via one spermatophore, to fertilise her full clutch. This relationship is investigated further in a study of sperm utilisation by female *T. vulgaris* in Chapter 6.

4.5.3 Are male gametic strategies during an encounter influenced by differential sperm supplies?

During an encounter, larger males and males in better reproductive condition are predicted to deposit a similar or higher number of spermatophores, each containing higher numbers of sperm, than smaller males or males in poorer reproductive condition. This study, in common with previous studies (Halliday 1974; 1975; Baker 1990a; Hosie 1992), failed to find any relationship between spermatophore score and male body size or male reproductive condition. Baker's (1990a) study may have failed to demonstrate these relationships because the newts were tested early in the season. The effects of differential sperm supply within the testes of individual males may not become apparent until later in the season, when sperm supply may constrain spermatophore production, in which case my study across the whole breeding season should have revealed any relationship between spermatophore score and body size. The lack of relationship between body size and spermatophore score suggests that larger males are unable to deposit more spermatophores during an encounter than smaller males, which may be a consequence of physiological constraints on the production of sperm accessory materials rather than a consequence of differential supplies of sperm (discussed below).

This study has found no relationship between the number of sperm per sperm mass, and either male body size or male reproductive condition. Taken together, the lack of correlation between body size and the number of sperm in a sperm mass, and between

body size and spermatophore score (Baker 1990a; this study) suggests that, if larger males do possess more sperm than smaller males, then their larger sperm supply may give a larger male smooth newt an advantage over a different time scale, such as the ability to produce more spermatophores throughout the whole season (investigated in chapter 5).

Spermatophore score, but not sperm number per sperm mass, was related to male body condition (at the time of capture from the pond), which is consistent with Baker's (1990a) study that found a positive, though non-significant trend, between spermatophore score and male body condition, and suggests that body condition may be an important determinant of spermatophore production (discussed further in chapter 5).

4.5.4 Are male gametic strategies constrained by the production of sperm accessory materials?

a) During a single encounter

The number of sperm in each sperm mass decreased significantly after the deposition of the first three spermatophores of an encounter, suggesting that the decrease may be due to reduced levels of the secretory products which form the matrix of the sperm mass or may be a consequence of the time taken for sperm to travel from the vasa deferentia to the site of packaging. It is likely that reduced levels of secretory products limit the number of sperm because I have shown that the pelvic glands of the cloaca, which produce the matrix of the sperm mass, require time to recover after spermatophore deposition (Chapter 3).

Decreasing sperm counts in successive ejaculates have been reported for a variety of species, e.g. voles (Pierce *et al.* 1990), and zebra finches (Birkhead *et al.* 1995). These studies have shown that transfer of reduced numbers of sperm does not lead to loss of potency, defined as the number of offspring produced from an ejaculate, suggesting that males of most species cease to mate before loss of potency occurs. However, later ejaculates were less likely to result in pregnancy (Austin & Dewsbury 1986), which may be a consequence of depleted sperm accessory materials rather than reduced sperm numbers. It is not yet known whether male newts also cease to produce spermatophores before loss of potency occurs.

Male reproductive success may still be limited even if potency is not affected by lower sperm numbers per sperm mass. Males that have only one spermatophore available for deposition are less likely to succeed in sperm transfer (Halliday 1974), which may be a consequence of females choosing not to pick up the spermatophore from males displaying at reduced rates (Halliday & Houston 1978), as females preferentially pick up spermatophores from males that display at a high rate (Teyssedre & Halliday 1986). The ability to discern a male's rate of display may enable females to avoid mating with depleted males that have a reduced sperm supply (Trivers 1972; Dewsbury 1982; Nakatsuru & Kramer 1982; Parker 1982). Similarly, females that elicit one or more spermatophores from a partially exhausted male, who may be displaying at a low rate, must be very highly motivated to mate (Hosie 1992), which may indicate that these females need to replenish their sperm supplies (discussed further in chapter 6).

Pick-up of sperm masses deposited later in an encounter may result in multiple insemination of the female. Therefore, sperm from these 'later' sperm masses can be considered to be 'additional sperm', increasing the number of sperm of that particular male in the female tract. In this case, the low sperm numbers in 'later' sperm masses may not be as important as when a female picks up just one sperm mass. Consequently, the reproductive success of the male may be enhanced through transfer of further sperm masses, despite the reduced numbers of sperm in each.

b) Throughout the breeding season

The number of sperm per sperm mass increased rather than decreased across the season, when all spermatophores were considered, irrespective of deposition order, refuting the hypothesis that the gametic strategies of males over the season may be constrained by their finite sperm supply (Verrell *et al.* 1986) or the production of sperm accessory materials as the cloacal glands regress (Sever *et al.* 1990). However, this relationship no longer held when the effect of deposition order (first to fourth) was considered, suggesting that the increase in the number of sperm per sperm mass with time in the season (when all spermatophores are considered), is due to an increase in the relative number of sperm

allocated to the fourth and subsequent spermatophores deposited during an encounter. The increase in the number of sperm in the fourth sperm mass as the season progresses may reflect larger reserves of sperm accessory materials. Males may experience limited mating opportunities in the wild towards the end of the season, which allows them more time to replenish the secretory products of their cloacal glands between matings. Similarly, the differential production of spermatophores during an encounter suggests that males depositing only one or two spermatophores may be constrained in the number of spermatophores that they can produce, due to either a reduced sperm supply or a reduction in sperm accessory materials. These findings suggest that any effect due to supplies of sperm accessory materials or sperm becoming limited during the season may be masked by differential mating opportunities in the pond, prior to testing. Therefore, the longitudinal study of males which investigates spermatophore production throughout one breeding season (Chapter 5), may reveal constraints of sperm supply or production of sperm accessory materials on sperm allocation.

4.5.5 Conclusions

The finding that male smooth newts are unlikely to be able to allocate sperm in response to the current sociosexual environment, but can maintain similar numbers of sperm in each sperm mass both during an encounter (consisting of three spermatophores) and throughout the season, suggests that male newts are exhibiting a maximal insemination strategy. This hypothesis is also supported by the observation that the number of sperm per sperm mass is sufficient, in numerical terms, to fertilise a full clutch. However, several factors in newt reproductive biology suggest that this strategy may be unlikely, and that males may be allocating sperm judiciously between the sperm masses.

During a courtship encounter a male smooth newt normally requires at least one tail-touch from the female to initiate each spermatophore deposition, suggesting that males must allocate sperm prudently to maximise their mating success (Halliday 1976). Yet in the complex environment of the pond, a male may not only inseminate the female that he is

courting, but he may be duped into wasting spermatophores by responding to the tail-touch of a rival male (Verrell 1984a), or he may deposit a spermatophore in response to one female that is picked up by a rival female (Waights 1996). Thus male newts may be unable to ascertain the fate of each individual sperm mass, which suggests that selection may favour males that minimise sperm wastage and maximise their reproductive potential by optimising the number of sperm in each sperm mass with the maximum production of spermatophores (see Fig 4.1). Markow (1985) proposed that the more accurately a polygamous male can predict that multiple mates will be available, the flatter the relationship between the number of sperm in successive ejaculates, but my findings suggest that, in smooth newts, the very flat relationship between the sperm numbers of the first three spermatophores deposited during an encounter may be a consequence of the uncertainty that deposition of a particular spermatophore will result in paternity, rather than a consequence of the predictability of gaining future matings. Such a strategy may reduce the number of sperm wasted when the female does not pick up the spermatophore, but may also reduce the number of offspring fathered by the male in the event of sperm competition. Thus the optimal number of sperm in a sperm mass will represent a trade-off between selection forces acting in these two opposing directions.

Taken together, the findings in this study, in conjunction with other studies, suggest that individual males may have differential sperm supplies, both before single encounters and in total for the season, but that the combined selection pressures acting on the male, due to multiple insemination of females during a single encounter, the inability to forcibly inseminate a female or to ascertain the fate of a particular sperm mass due to sexual interference and female choice, and from multiple mating by females throughout the season, may favour sperm allocation according to a bet-hedging strategy. Males may maximise their reproductive potential by optimising the number of sperm per sperm mass with the number of spermatophores available for deposition, a strategy which maximises spermatophore production while minimising sperm wastage.

4.6. Summary

The number of spermatophores deposited by individual male smooth newts, tested once only during a breeding season, was not related to male body size or reproductive condition (tail height), but was related to body condition.

The number of sperm per sperm mass deposited by individual males, tested once only during a breeding season, showed considerable variation, from 38 000 to 148 000, but was not correlated with body size, body condition or reproductive condition (tail height). These relationships will be further elucidated in chapter 5, when spermatophore production in males of varying body size will be investigated.

Each sperm mass, deposited by males during a single encounter or during single encounters across the season, contained sufficient sperm, in numerical terms, to fertilise a full clutch. This relationship is investigated further in a study of sperm utilisation by female *T. vulgaris* (Chapter 6).

The decrease in sperm numbers contained in sperm masses deposited later in an encounter is probably a consequence of physiological constraints on spermatophore production.

The sperm conservation hypothesis does not adequately predict the gametic strategy of male smooth newts observed in this study. The data obtained in this study suggests that males may allocate sperm to sperm masses in accordance with the bet-hedging hypothesis. Two further longitudinal studies, namely spermatophore production in individual males throughout the season (Chapter 5) and sperm utilisation in female smooth newts (Chapter 6) aim to elucidate this further.

Chapter 5. Male body size and reproductive success in the smooth newt

5.1. Introduction

The reproductive success of male animals is generally dependent on the number of mates that an individual obtains rather than the number of gametes he produces (Bateman 1948; Trivers 1972). Therefore, male reproductive success is expected to show greater variance than female reproductive success, which has been demonstrated empirically, e.g. in the salamander *Desmognathus ochrophaeus* (Houck *et al.* 1985b; see also Clutton-Brock 1988 for general review). The most informative measure of reproductive success is lifetime reproductive success, defined as the number of offspring reaching sexual maturity and themselves reproducing that are produced by an individual throughout his or her lifetime. A few studies have succeeded in calculating how many offspring of particular individuals enter the breeding population, notably, the sixteen year study of the red deer population on Rhum (Clutton-Brock 1988), but this is difficult to determine for many species due to their long reproductive lifespan and the delay, often of several years, before the onset of sexual maturity in the offspring.

Consequently, several partial components have been identified which can give an indication of the reproductive success of an individual, for example, number of:- spermatophores produced (reproductive potential), mates acquired (mating success), zygotes fertilised (fertilisation success), offspring born or hatched (hatching success), or offspring surviving to sexual maturity (recruitment success). Any one partial component is often a poor

predictor of the contribution of other partial components, e.g. mating success is often poorly correlated with fertilisation success and hatching success (Clutton-Brock 1988). Thus measurement of several of these parameters in an individual may give a closer approximation of overall reproductive success, although chance factors may contribute significantly to some partial components, such as reproductive life span or number of mates encountered (Clutton-Brock 1988).

Clutton-Brock (1988) identifies four principal questions relating to reproductive success: how widely does reproductive success vary between individuals? how much of the variance in success is contributed by the different components of reproductive success? to what extent does reproductive success change with age? and what environmental, genetic, developmental or phenotypic factors affect reproductive success? This study is concerned with the last question, namely, to what extent does adult body size influence male reproductive success?

5.1.1. Body size

Adult body size is influenced by life history traits and, therefore, is determined by a combination of genetic and phenotypic factors. Larger body size may be favoured in unpredictable environments due to the positive correlation between fasting endurance and body size (Millar & Hickling 1990; 1992), although Speakman (1992) suggests that, even in unpredictable environments, selection may be acting independently during periods of food abundance and scarcity to give correspondingly different optimal body sizes. Much of the variation in adult amphibian body size is due to phenotypic expression in response to varying environments, during both larval and juvenile periods of growth, e.g. larvae grow larger at low density in *Notophthalmus viridescens* (Harris 1987), at lower temperatures in *Rana pipiens* (Smith-Gill & Berven 1979), and if produced early in the season in *Ambystoma talpoideum* (Semlitsch *et al.* 1988). Some heritability of the trait has also be found, e.g. in *Bufo woodhousei*, larger males produce offspring that are larger at metamorphosis (Mitchell 1990), but a similar study in *Bufo bufo* failed to find any relationship (Semlitsch 1994).

In species that exhibit indeterminate growth, larger body size may be a consequence of survival to an advanced age (Halliday & Verrell 1988). Age is significantly correlated with body size in many urodeles, including *T. vulgaris* (Verrell & Francillon 1986), but males of similar body size may vary two-fold in age. Both faster growth rates and long-term survival ability are signs of phenotypic vigour and, if larger body size is heritable, larger body size may indicate a superior genotype.

Larger body size may also be a consequence of selection for components of reproductive success, such as larger energy reserves (which may increase success in scramble competition) or enhanced survival of offspring, e.g. in the anurans *Scaphiopus couchi* (Woodward 1987) and *Hyla crucifer* (Woodward & Travis 1991). Males generally compete for access to mates and a large body size may enhance male reproductive success, by giving a male an advantage in either male-male competition or in his ability to attract mates. Larger males have been shown to achieve higher reproductive success in many species via both of these mechanisms (Clutton-Brock 1988; Andersson 1994). In urodeles, evidence for larger males achieving higher reproductive success is sparse (Table 5.1). Fighting among males, in the form of biting, has only been observed in a few species in the laboratory (Sparreboom 1984; Raxworthy 1989; Thiesmeier & Hornberg 1990; Verrell & Donovan 1991; Halliday & Tejedo 1995). For example, in *Triturus vittatus*, fighting is thought to be related to territorial defence but there is no evidence that larger males are more successful (Raxworthy 1989). In *Desmognathus ochrophaeus*, larger males are able to prevent smaller males from gaining access to receptive females (Houck 1988; Verrell & Donovan 1991) and large male *Notophthalmus viridescens* can displace small males during courtship (Verrell 1986b). In contrast to these studies, there is no evidence to suggest that larger males have an advantage in either sexual interference or sexual defence (Halliday & Verrell 1986; Halliday & Tejedo 1995), although in the laboratory *T. vulgaris* females prefer to remain in the vicinity of males of comparable body size (Verrell 1991a).

In *Triturus cristatus*, larger body size results in higher mating success, but this relationship is only observed prior to the development of the crest (Hedlund 1990). The advantage due to

larger body size thus occurs at the beginning of the season, and may only partially increase reproductive success since congeneric female *T. vulgaris* cannot lay an entire clutch from one insemination (Chapter 6). Houck & Francillon-Vieillot (1988) found that seasonal reproductive potential (number of spermatophores deposited) was not correlated with either body size or age within a specific size-class in male *Desmognathus ochrophaeus.*

Table 5.1 The effect of male body size on components of reproductive success in urodeles.

	species	**relationship**	**reference**
Reproductive potential			
spermatophore production during a single encounter	*Triturus vulgaris*	no relationship	Baker 1990a Chapter 3, 4
	Desmognathus ochrophaeus	no relationship	Houck *et al.* 1985b
	D. ochrophaeus	no relationship	Houck & Francillon-Vieillot 1988
spermatophore production during repeated encounters	*T. carnifex*	no relationship	Malacarne & Cortassa 1983
testes size	*T. vulgaris*	positively correlated	Verrell *et al.* 1986
Mating success			
intermale competition	*D. ochrophaeus*	large males prevent small males gaining access to mates	Houck 1988
	Notophthalmus viridescens	Large males displace small males	Verrell 1986b
spermatophore pick-up success during a single encounter	*T. cristatus*	females preferentially pick-up spermatophores from large males before crest has developed fully	Hedlund 1990
	D. ochrophaeus	no relationship	Houck *et al.* 1985b
	T. vulgaris	no relationship	Green 1991 Hosie 1992
spermatophore production during repeated encounters	*T. carnifex*	no relationship	Malacarne & Cortassa 1983

5.1.2. Male body size and female choice

Females choosing to mate with fitter males may gain 'good genes' to pass on to their offspring (Williams 1966; Halliday 1978). Body size is frequently used as a measure of fitness because in many species size tends to increase with age (and, therefore, with

survival) and larger individuals often achieve higher reproductive success, both of which are partial components of fitness (Vehrencamp & Bradbury 1984). Thus females are predicted to mate preferentially with larger males, whose size indicates enhanced survival and a superior genotype (Trivers 1972). A possible mechanism for this genetic correlation may be via deleterious mutations which cause less fit individuals to die at a younger age (Manning 1985). This view has been challenged recently by Hansen & Price (1995) who propose that older males may rarely be genetically superior for the following reasons:- i) there may be negative genetic correlations between late-age and early-age life history parameters; ii) deleterious germ-line mutations increase with age and decrease male fitness; iii) mean population fitness may increase with generation and iv) the covariance between fertility and fitness may decrease with age, so that gametes from older males may have lower viability. The authors' proposals are supported by empirical evidence in the first two cases only. Nevertheless, the authors conclude that, contrary to current opinion, females choosing to mate with larger, older individuals are not providing evidence to support the good genes models but are providing evidence against them. These proposals need further investigation because, although these hypotheses could explain why selection would act against females choosing to mate with very old individuals, it seems implausible to suggest that females gaining no direct benefits other than genes from a mating would choose to mate with an older, genetically inferior, male.

5.1.3. Epigamic characters and female choice

Although there is little evidence for a correlation between male body size and male reproductive success in urodeles, there are several studies that show a relationship between the degree of development of the tail and crest (male epigamic characters) and male reproductive success (Table 5.2). These studies suggest that male reproductive success is more influenced by tail height attained than by absolute body size. Halliday (1978) proposed that males with fully developed epigamic characters may be fitter. Thus fully developed characters, such as the dorsal crest of the smooth newt, may be 'viability indicators'

(Andersson 1982; 1986; 1994; Bradbury & Andersson 1987), enabling females to mate selectively with superior males.

Table 5.2 Studies that demonstrate a relationship between the degree of development of male epigamic characters and components of male reproductive success.

component	character	species	reference
reproductive potential			
proportion of testes that has produced sperm	positively correlated with tail height	*Triturus vulgaris*	Verrell *et al.* 1986
number of spermatophores deposited	positively correlated with tail height	*Notophthalmus viridescens*	Verrell 1982b
mating success			
spermatophore pick-up success during single or repeated encounters	females preferentially pick-up spermatophores from males with larger crests or tail heights	*T. cristatus* *T. carnifex* *T. vulgaris* *N. viridescens*	Hedlund 1990 Malacarne & Cortassa 1983 Green 1991 Hosie 1992 Verrell 1982b
	females mate sequentially with males with higher crests	*T. vulgaris*	Gabor & Halliday (1997)

The development of sexual characters is thought to be costly, since males of many species only develop ornaments during the breeding season (Andersson 1982; Halliday 1990a). Andersson's (1982) models of selection and quality advertisement, based on the 'handicap hypothesis' of Zahavi (1975; 1977), predict that optimal ornament size should increase with phenotypic quality, a prediction which is also proposed by other authors (Halliday 1978; Williams 1978). Phenotypic quality may differ in response to many factors, such as parasite load (Hamilton & Zuk 1982) or nutritional status (Clutton-Brock *et al.* 1988). Anderson (1986) extended this hypothesis and proposed that females may choose mates on the basis of traits that are costly for a male to exhibit. Only males in good condition are able to produce the trait to its fullest extent; thus females choosing to mate with highly ornamented males are choosing to mate with males in good condition. Andersson's (1982) hypothesis assumes that condition and expression of the trait are highly correlated and that the two factors are heritable. Tail depth has been shown to be condition dependent in both *T. vulgaris* (Green

1991) and *T. cristatus* (Baker 1992b) and condition has a genetic component in many domestic animals, e.g. in pigs (Smith *et al.* 1962). Condition may also have a genetic component in smooth newts.

It is difficult to determine whether male newts with larger crests achieve higher reproductive success because they are more attractive to females, which may also be influenced by their higher testosterone levels and higher display rate (Malacarne & Cortessa 1983), or because they have a larger number of spermatophores available. Baker (1990a) found no relationship in *Triturus vulgaris*, between tail height and reproductive potential, suggesting that tail height is not a good indicator of the availability of spermatophores, or between male body size and reproductive potential. Therefore, any advantage of larger body size in terms of mating capacity must act over a different time scale, such as throughout the annual breeding season or over the whole breeding lifetime of an individual (Halliday 1987), or in a way not directly related to the number of spermatophores produced, such as a longer seasonal reproductive period.

5.2. Aims

The study comprises two sections. Section 5.3 will investigate the effect of body size on male reproductive potential, measured as spermatophore production throughout a single breeding season. Section 5.8 will investigate whether males have the capacity to mate multiply during a single reproductive episode, similar to the narrow window of daily mating opportunity (Griffiths 1985), which does not give males sufficient time to replenish their spermatophore supplies between encounters. This study will give an indication of the relationship, if any, between body size and potential reproductive success in male smooth newts during a single evening and throughout an entire breeding season.

5.3. Seasonal spermatophore production

The study will test the following hypothesis:-

Hypothesis H_1: Larger males exhibit higher reproductive potential during a breeding season than smaller males.

Prediction 1: Larger males deposit a higher number of spermatophores, in total, over the course of a breeding season than smaller males, enabling them potentially to inseminate more females.

Prediction 2: Larger males deposit spermatophores for a longer period in the breeding season than smaller males, giving them mating opportunities later in the season.

5.4. Methods

5.4.1. Collection of newts

Unmated newts were collected on land during the spring migrations to the breeding ponds in 1993 and 1994 and were maintained as described previously (sections 2.2 and 2.3). Twelve males in 1993 and twenty males in 1994 were selected to include a range of body sizes (snout-vent length [svl] =39 to 51 mm). Each individual was coded by toe-clipping for future recognition and the following morphometrics were recorded; snout-vent length [svl], tail depth [td], and weight [wt] as described in Chapter 2, section 2.4.3. In natural populations, the degree of development of the crest is highly correlated with the height of the tail fin (Baker 1990a). Therefore, tail height was used as a measure of the degree of development of the secondary sexual characters.

5.4.2. Spermatophore production

The spermatophore production of each male across the whole breeding season was determined using a series of standardised courtship encounters (described in Chapter 2, section 2.4.5). Male newts require a 24-48 h recovery period after courting to sexual exhaustion (Verrell 1986a). In Chapter 3, I established that the recovery period is related to

replenishment of the secretory products in the cloacal glands, which produce the spermatophore base and the matrix for the sperm mass. In this study, each male was tested twice weekly, to ensure spermatophore production was not limited by insufficient replenishment of the secretory products of the cloacal glands.

Once a week, the following measurements were taken; [svl], [td] and [wt] as described above. After each test, the males were returned to their holding tanks. The males were tested throughout the season until they all had ceased spermatophore production. By this time, the males were showing morphological changes commensurate with returning to the terrestrial habitat. Their dorsal crests had regressed significantly and their skin had reverted to the terrestrial condition.

5.5. Results

Two males in 1993 and three males in 1994 deposited spermatophores on the first day of testing only, so they have been excluded from the analysis.

5.5.1. Spermatophore production by individual males

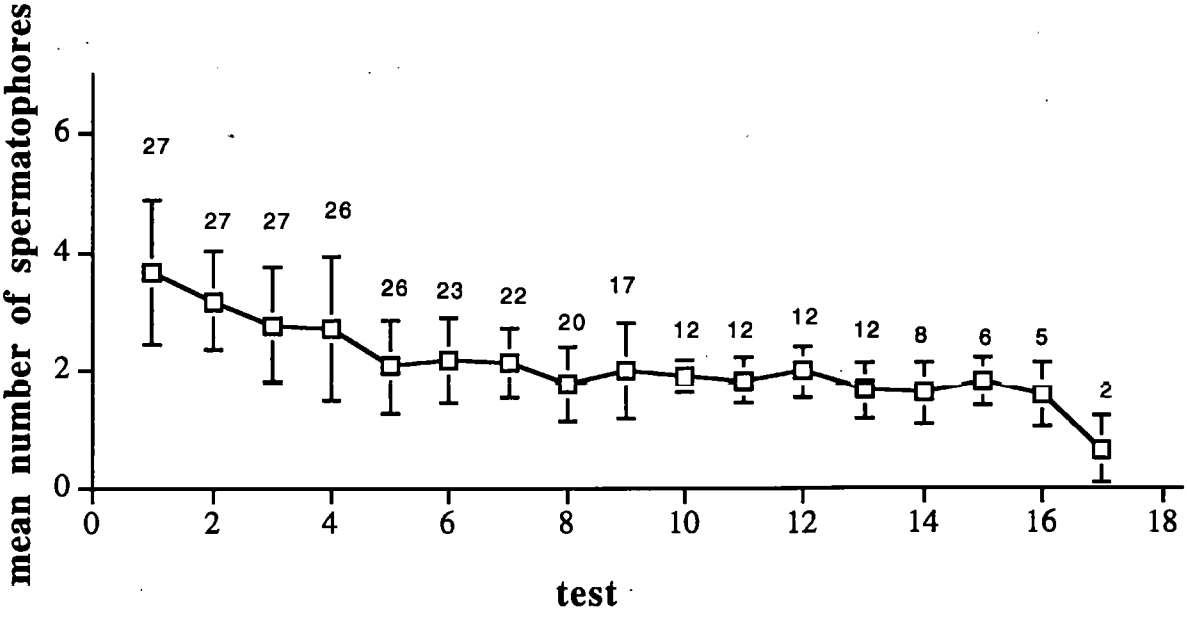


Fig 5.1 Pattern of the number of spermatophores deposited in each test (mean±s.d) by individual males during a longitudinal seasonal study (Test 1=first test in which each male deposited spermatophores, all tests corrected to be consecutive, see text for details. Numbers above each mean=number of males contributing to that point).

The number of spermatophores deposited by individual males during a standardised courtship encounter declined as the season progressed (Fig 5.1), which is consistent with the pattern of deposition observed in *T. vulgaris* by Halliday (1976). Some males, mainly those with low seasonal spermatophore production, were unable to deposit spermatophores in every test. To compare spermatophore production in consecutive tests, the encounters in which no spermatophores were deposited were ignored in Fig 5.1. Student t-tests revealed that the number of spermatophores deposited by males during their first test of the season was significantly greater than the number deposited during the fifth test in which spermatophore deposition occurred (test1:test 5; $t=0.53$, $p<0.0001$). However, after this initial reduction in spermatophore number, the number of spermatophores deposited during each test was fairly constant over the rest of the season (test5:test14; $t=1.50$, $p=0.071$). Similar declines in the spermatophore production of individual males during a breeding season have also been observed in *Desmognathus ochrophaeus* (Houck *et al.* 1985b).

The DNA of sperm masses deposited throughout the season was determined (method described in Chapter 4) for males in the 1993 study.

Table 5.3 Comparison of the amount of DNA in sperm masses produced by individual males throughout the 1993 breeding season. The DNA content of spermatophores deposited by the males during weeks 2-7 was compared to the DNA content of spermatophores deposited in week 1 using repeated measures ANOVA (SPSS). n=10 males; $F_{6,12}=10.556$; $p=0.0003$; *=significant difference between the two means, $p<0.05$).

week	number of spermatophores (one per male) in each group	DNAx10^{-6} g mean±s.d.	F	p
1	10	1.63±0.23		
2	9	1.42±0.29	6.88	0.11
3	9	1.45±0.23	13.80	0.065
4	7	1.44±0.25	20.83	**0.045***
5	4	1.14±0.09	21.88	**0.043***
6	4	1.12±0.81	23.65	**0.040***
7	3	0.98±0.13	30.11	**0.031***

To investigate whether the amount of DNA deposited by individual males decreases across the season, the sperm mass containing the most DNA deposited by a male in a given week was compared with the sperm mass containing the most DNA deposited by the same male during the first week using repeated measures ANOVA (Table 5.3). This comparison revealed that sperm masses deposited early in the season contained significantly more DNA than those deposited late in the season This result is discussed in Chapter 8.

5.5.2. Comparison of the data from the two breeding seasons

There was no difference between the snout-vent length [svl] of the two groups (1993: n=10, mean [svl]= 46.9±3.22 mm; 1994: n=17, mean [svl]= 45.6±3.09 mm; t-test: t=1.13, p=0.269). However, the difference between the weights [wt] of the two groups was close to significant (1993: mean [wt]=2.56±0.42 g; 1994: mean [wt] =2.23±0.42 g; t-test: t=2.00, p=0.06).

Two methods were evaluated to determine the body condition of males on arrival at a pond. The 'residual' method (Baker 1990a; Stearns 1992) determines body condition from the residuals of the regression of weight [wt(g)] on snout-vent length [svl^3(mm)]. Positive residuals indicate animals in better condition than average, and negative residuals indicate animals in poorer than average condition. The 'absolute value' method (Bonetti 1996, adapted from Veith 1987) determines body condition by calculating [wt(g)]/[svl^3 (cm)]*1000. The 'absolute value' method enables estimation of the body condition of a single individual, whereas the 'residual' method requires sufficient individuals to calculate a regression. A potential drawback of the 'absolute value' method is that, because it assumes that mass increases as a cubic function of snout-vent length, any deviation from this relationship due to allometric growth may introduce bias. To check that the [wt]/[svl^3]*1000 relationship holds across the range of snout-vent length, the body condition (absolute value) on arrival at a pond was calculated for 100 newts ([svl]: range = 38 - 52 mm). The newts were grouped for each 3 mm increase in snout-vent length (the two smallest groups contained few individuals, so these groups were combined to give one group containing

individuals with snout-vent lengths from 38 - 43 mm) and the mean body condition was calculated for each group. The mean body conditions for each group were compared by the LSD test for multiple comparison of means, which revealed significant differences between the mean body condition of males in the smallest snout-vent length class and the mean body conditions of two other size classes (Table 5.4). This relationship also holds if the mean body condition of all the newts larger than those in the smallest class is compared with the mean body condition of males in the smallest size class (student's t-test: $n_1=14$, $n_2=86$, df=98, t=3.043, p=0.002).

Table 5.4. Comparison of the mean body condition of newts grouped by snout-vent length [svl]. (LSD test for comparison of multiple means: n=number of males; $F_{4,97}=4.18$, p=0.008. The means of ranges 44-46 and 47-49 differ significantly from range 38-43 ($p<0.05$). There are no other significant differences.).

svl range (mm)	**n**	**body condition (mean±sd)**
38-43	14	24.91±3.06
44-46	31	23.12±2.28
47-49	38	22.29±1.85
50-53	18	23.25±2.92

Consequently, the 'absolute value' method may be suitable only to compare body condition in individuals of similar snout-vent length. The 'residual' method estimates average condition throughout the range of body size, reducing any deviation from the relationship due to allometric growth. Therefore, the 'residual method' was used to determine body condition in newts used in this study.

When the body condition of the newts entering the pond is plotted against male snout-vent length, the number of males in poor condition (below the zero line) is higher than the number of males in good condition (above the zero line) in 1994, whereas the opposite is true in 1993 (Fig 5.2). Therefore, combining the data collected during the two study years will enable seasonal spermatophore production to be determined over a range of body size in conjunction with a range of body condition. To ensure that this approach is valid the two data sets were also analysed separately.

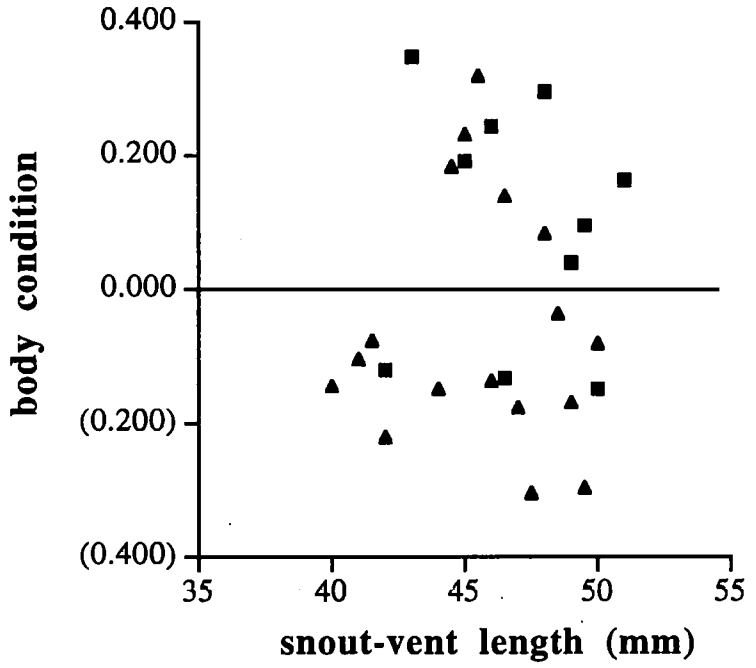


Fig 5.2 Comparison of body condition on arrival at the pond for males collected in 1993 ■, and in 1994 ▲. Line indicates males in average condition for their snout-vent length (numbers in brackets are negative).

5.5.3. Prediction 1: *Larger males deposit more spermatophores throughout the breeding season than smaller males*

Spermatophore production across the season in each year was correlated with both male body size parameters, snout-vent length and weight (Table 5.5), although in 1994 the positive trend between spermatophore production and weight was not statistically significant (p=0.06).

Table 5.5 Relationship between the total number of spermatophores deposited during the breeding season and various male body parameters: snout-vent length [svl]; weight [wt]; maximum tail height attained during the season [maxtd]; body condition [body cond^n]; change in body condition during the season [%change] (1993: n=10, df =9; 1994: n=17, df=16; Pearson product moment correlations: *p<0.05; **p<0.01; ***p<0.005).

	svl	wt	body cond^n	%change	maxtd
1993					
r	0.66*	0.62*	-0.36	0.58	0.74**
1994					
r	0.48*	0.46	-0.11	-0.16	0.70***
1993/4					
r	0.55**	0.48*	-0.18	0.28	0.66***

Spermatophore production across the season, in each year, was also correlated with the maximum tail depth that a male achieved during the breeding season. Maximum tail depth was determined because tail depth increases as the breeding season progresses (Griffiths & Mylotte 1988). The maximum tail depth that a male achieved during the season was also positively correlated with his snout-vent length ($n=27$; $df=26$; $r=0.53$, $p<0.001$).

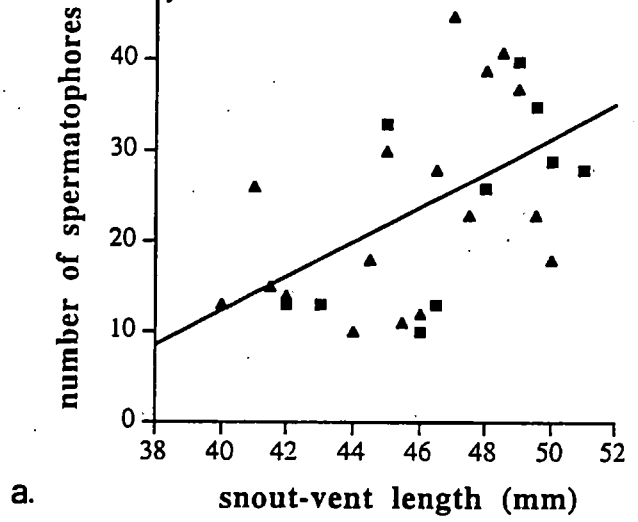
Two other factors may also influence seasonal spermatophore production; body condition on arrival at the pond and percentage change in body condition during the breeding season. Baker (1992b) has shown, in male *Triturus cristatus*, that maximum crest height achieved during the season is correlated with their body condition on entering the water. The body condition of an individual on migration may reflect his foraging ability during the terrestrial phase, or may simply reflect environmental conditions during that particular winter. Similarly, the percentage change in body condition during the breeding season may reflect the foraging ability of an individual during the aquatic phase.

Pearson product moment correlations showed that body condition on arrival at the pond and the percentage change in body condition during the breeding season were not correlated with the number of spermatophores produced during the season (Table 5.5). Stepwise multiple regression was carried out to establish which male parameters are the best predictors of seasonal spermatophore production (analysing data from each year separately and combining the data for the two years, Table 5.6).

The analysis revealed that the maximum tail depth attained by an individual during the season was the best predictor of spermatophore production during the breeding seasons of 1993 and 1994 (and for the two years combined). The relationships between spermatophore production and the various body parameters (data combined for the two years) are shown in Fig 5.3.

Table 5.6. Stepwise multiple regression of parameters that may predict the total number of spermatophores deposited by an individual male during one breeding season. The table shows regression models after variables are removed from the equation, in turn, in order of least significance. Two sets of coefficients and p values are given: (i) taking into account all the parameters, and (ii) after stepwise regression including only those variables which are significant ($p<0.05$). (snout-vent length [svl], maximum tail depth achieved during the season [maxtd], body condition [con], change in body condition during the season [%change], *$p<0.05$,**$p<0.01$, ***$p<0.005$).

1993 (n=10)	**SVL**	**maxtd**	**con**	**%change**	**intercept**	**r^2**	**p**
i) coefficient	1.078	2.310	8.396	0.0123	-49.720	0.77	0.073
p	0.27	0.158	0.557	0.157	0.225		
ii) coefficient		3.91			-13.036	0.55	**0.014***
p		0.014			0.314		
1994 (n=17)							
i) coefficient	-0.05	6.887	-17.53	-0.006	-35.094	0.59	**0.02***
p	0.957	0.008	0.218	0.309	0.343		
ii) coefficient		5.8			-28.494	0.49	**0.002****
p		0.0018			0.059		
1993/1994 (n=27)							
i) coefficient	0.723	4.136	-7.751	-0.001	-47.44	0.53	**0.002****
p	0.307	0.0033	0.403	0.793	0.091		
ii) coefficient		4.623			-18.65	0.48	**0.0001*****
p		0.0001			0.0489		



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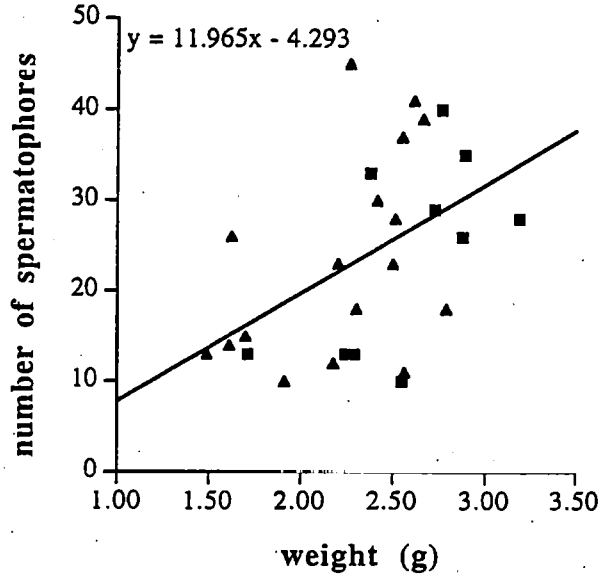

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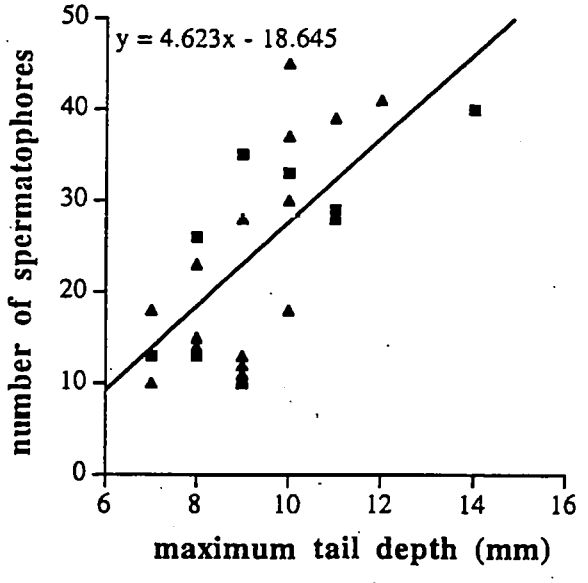


c.

Fig 5.3 Relationship between the total number of spermatophores produced during the breeding season and various male body parameters: a) snout-vent length, b) weight, and c) maximum tail height attained during the season (data for the two years combined: n=27; df=26; 1993: ■; 1994: ▲).

5.5.4. Prediction 2: *Larger males deposit spermatophores for a longer period during the breeding season than smaller males*

In 1993, the length of the period, measured in days, during which males deposited spermatophores (deposition period) was correlated with both male body size parameters, snout-vent length and weight, and with the maximum tail height that a male achieved during the season (Table 5.7). However, in 1994, when many males were in comparatively poor body condition (Fig 5.2), the length of the deposition period was correlated only with the maximum tail height that a male achieved during the season (Table 5.7).

To investigate which of the above relationships best predicts the duration of the deposition period, stepwise multiple regression was carried out (analysing data from each year separately and combining the data for the two years, Table 5.8).

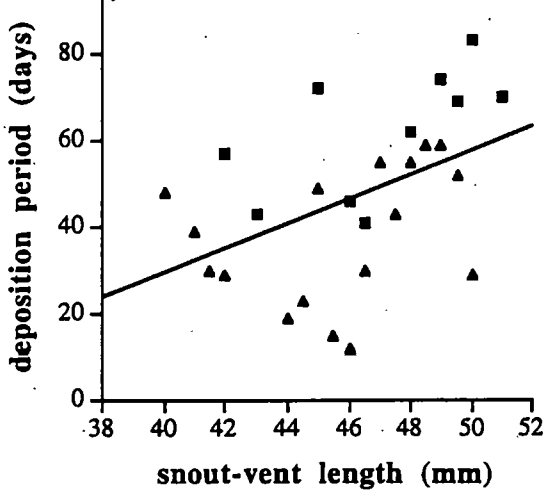
Table 5.7 Relationship between the duration of the deposition period and various male body parameters: snout-vent length [svl]; weight [wt]; maximum tail height attained during the season [maxtd]; body condition [body cond^n]; change in body condition during the season [%change]; (1993: n=10, df =9; 1994: n=17, df=16; Pearson product moment correlations: *p<0.05; **p<0.01).

	svl	wt	body cond^n	%change	maxtd
1993					
r	0.59*	0.50*	-0.17	0.34	0.73**
1994					
r	0.32	0.21	-0.11	0.26.	0.58**
1993/4					
r	0.46*	0.45*	-0.07	-0.05	0.58**

Stepwise multiple regression revealed that the maximum tail depth attained by an individual during the breeding season was the best predictor of the duration of the deposition period, although this relationship was weaker than the relationship between maximum tail depth and the number of spermatophores produced. There was also a relationship between the number of spermatophores produced and the duration of the deposition period (n=27, df=26, r=0.44, p=0.0002). The relationships between the various male body parameters and the deposition period (data combined for the two years) are shown in Fig 5.4.

Table 5.8. Stepwise multiple regression of parameters that may predict the duration of the deposition period exhibited by an individual male during one breeding season. The table shows regression models after variables are removed from the equation, in turn, in order of least significance. Two sets of coefficients and p values are given: (i) taking into account all the parameters, and (ii) after stepwise regression including only those variables which are significant ($p<0.05$). (snout-vent length [svl], maximum tail depth achieved during the season[maxtd], body condition [con], change in body condition during the season [%change], * $p<0.05$).

1993 (n=10)	**SVL**	**maxtd**	**con**	**%change**	**intercept**	**r^2**	**p**
i) coefficient	1.325	3.810	-12.199	0.0021	-36.867	0.61	0.24
p	0.417	0.173	0.618	0.881	0.577		
ii) coefficient		5.078			13.460	0.53	**0.017***
p		0.0173			0.437		
1994 (n=17)							
i) coefficient	0.528	7.547	0.159-	0.009	-55.012	0.51	0.054
p	0.726	0.041	30.738	0.364	0.327		
ii) coefficient		6.733			-22.6	0.34	**0.014***
p		0.0014			0.320		
1993/1994 (n=27)							
i) coefficient	1.094	6.276	-22.036	-0.001	-61.235	0.41	0.016
p	0.426	0.018	0.227	0.890	0.254		
ii) coefficient		6.737			-15.106	0.34	**0.015***
p		0.015			0.398		



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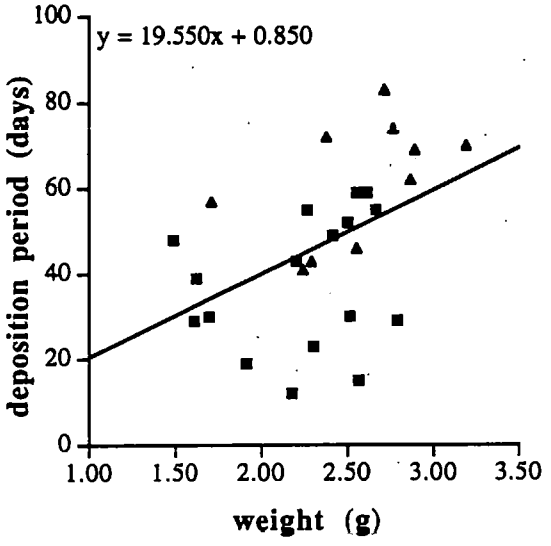

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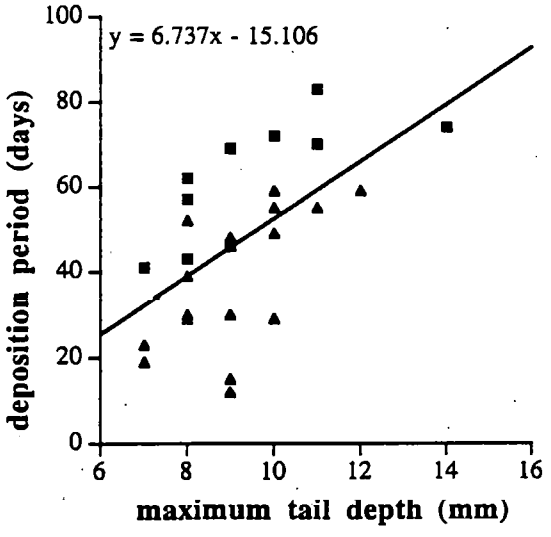


c.

Fig 5.4 Correlation between the duration of the deposition period and various male body parameters: a) snout-vent length; b) weight and c) maximum tail height attained during the season (data for the two years combined: n=27; df=26; 1993: ■; 1994: ▲).

5.6. Discussion

5.6.1. Can larger males deposit more spermatophores during the breeding season than smaller males?

This study shows that there was a positive correlation between spermatophore production over the season and male body size, with respect to both snout-vent length and weight. Nevertheless, the best predictor of spermatophore production over the season was the maximum tail depth attained by an individual. Tail depth is positively correlated with body size in smooth newts (Green 1991; 1992; Baker 1990a; this study), suggesting that one advantage of larger body size may be the ability to achieve well-developed epigamic characters while maintaining a good supply of spermatophores. However, despite positive allometry between tail height and snout-vent length in males captured in the wild (Green 1992; Baker 1990a), there is large variation in the tail height achieved by males of a similar size (Baker 1990a, this study), suggesting that absolute body size is not the only determinant of crest height.

Males may need to be in good body condition to realise their potential maximum crest height and spermatophore production, which may explain why some smaller males can achieve higher tail depths and higher reproductive potential than some larger males. For example, a male of 45 mm snout-vent length put down 33 spermatophores and achieved a tail depth of 10 mm, whereas a male of 48 mm snout-vent length put down 26 spermatophores and achieved a tail depth of 8 mm. A trade-off between tail depth and spermatophore production occurs in smooth newts (Halliday, Waights & Hosie in prep). Male newts fed a restricted diet maintained their crest height with respect to males fed *ad libitum* but reduced their spermatophore production. However, males still exhibited a range of tail depth in both groups, demonstrating that crest height was not determined by current food availability alone. This contrasts with Green (1991), who found that males brought in from the pond and subsequently starved lost more tail height than fed males. He interpreted this as tail

height being an honest indicator of current foraging intake, but this result could be confounded by stress. In our study male newts were collected on land and allowed to develop their crests under semi-natural conditions, which mimicked ponds with either high or low levels of resources. In Green's study the newts were captured from the ponds and brought into the laboratory, which is known to cause rapid regression of their crests and cessation of courtship behaviour (Wimpenny 1951; Verrell 1982c; Halliday pers comm). The loss of reproductive condition is attributed to stress, as stressed animals switch off reproduction via the same mechanisms that prevent reproduction under adverse environmental conditions (Moore & Miller 1984; Moore & Zoeller 1985; Greenberg & Wingfield 1987; Whittier 1991; Zerani & Gobbetti 1993). A more likely indication that crest height reflects phenotypic vigour and current foraging intake is another observation by Green (1991) that, for a given snout-vent length, heavier males in a population achieve a higher tail.

In my study, males were fed *ad libitum* during the breeding season to ensure sufficient current resources for maximum development of the crest and maximum spermatophore production. Despite this, the males achieved differing levels of spermatophore production and development of the crest, suggesting that crest height may reflect differential assimilation of food or that it is influenced by other factors in addition to current food intake. This variation may be a consequence of varying parasite load (Hamilton & Zuk 1982), although little is known regarding urodele parasites (Avery 1971; Elkan 1976) and only a few studies have investigated the effects of amphibian parasites on aspects on reproduction, e.g. in *Hyla versicolor* (Hausfater *et al.* 1990). The variation in development of the crest may reflect differential stored resources as individual males emerge from winter torpor with differing body condition (shown in *Triturus cristatus*, Baker 1992b). However, body condition on migration did not predict future spermatophore production, implying that males arrive at the pond in an internal condition that determines spermatophore production. Consequently, body condition may be more important during the latter part of the previous summer, when males are undergoing spermatogenesis. Verrell *et al.* (1986) found that testes weight, fat body weight and liver

weight increase during late summer, and that the variation among individuals is much greater at this time than in the spring. The fat body weighs significantly more during the autumn than on migration, and continues to decrease in size throughout the breeding season, suggesting that this energy store is used for maintenance during winter torpor, and to provide lipid precursors for the hormones and prostaglandins that are involved in reproduction. The cloacal glands increase in size during the winter, especially the dorsal gland (Verrell *et al.* 1986) that produces pheromone. Lipid precursors may be required for this synthesis as pheromone is known to be a progesterone-like substance in *T. carnifex* (Belevedere *et al.* 1984). In contrast to the fat body, liver weight is maintained throughout the winter but decreases rapidly during the breeding season, suggesting that the stored glycogen is utilised for the energetic demands of courtship display and to replenish the sugar moieties of the secretions of the cloacal glands. This demand for sugar moieties suggests that spermatophore production may be influenced by the type of food available. Males able to gain sugar moieties from either frog's eggs or from the glycogen-rich liver of tadpoles (Griffiths 1996) may be able to replenish their spermatophores more quickly or for a longer period than males feeding only on invertebrates, but this awaits investigation.

The proportion of glandular tissue, as opposed to spermatogenic tissue, in the testes is correlated with the tail height achieved during the breeding season (Verrell *et al.* 1986). As discussed in Chapter 1, the glandular tissue represents the portion of the testes that has released mature sperm into the vasa deferentia. Thus males that develop a high crest may also have a high number of stored sperm, a hypothesis which appears to be supported in this study as maximum crest height is a predictor of the total number of spermatophores deposited during a season. Smaller males in good condition in late summer may be able to produce large quantities of sperm. Conversely, larger males in poorer condition may only be able to produce lower levels of sperm, despite having larger testes. Body condition on migration may thus reflect environmental effects on stored resources, such as the severity of the winter, rather than the foraging ability of males during the terrestrial phase, which is supported by the finding that the proportion of males in good or poor condition on arrival

at the pond differed between the two years under study. Whether all the sperm produced in the autumn can be utilised as spermatophores may depend on the availability of food during the breeding season. Although the majority of males (25 out of 27) lost weight during this study, which is similar to the situation in the wild at the end of the breeding season (Verrell & Halliday 1985), the percentage change in body condition was not correlated with the number of spermatophores produced or with initial body condition. This finding is in contrast to the study in chapter 4, which found that spermatophore production during single encounters throughout the season was related to body condition. These conflicting results may reflect differential levels of food availability between the two types of study. Newts in the pond will be competing with adult newts, newt larvae and individuals of other carnivorous species for resources, whereas the males in the experimental studies were fed *ad libitum*, which may mask any effects resulting from differential body condition. Taken together, these findings suggest that body condition is an important factor determining individual male reproductive success.

The results of this study and the study by Halliday, Waights & Hosie (in prep), suggest that males trade off crest height and spermatophore production using both stored and current resources (Fig. 5.5). Under optimal food availability, larger males may produce both larger crests and more spermatophores than smaller newts. However, larger males in poorer condition may produce a reduced crest and fewer spermatophores than a smaller male in good condition. Alternatively, older males of each body size may develop larger crests and exhibit higher reproductive potential because, unlike younger males, they may invest more in current than in future reproduction, diverting a greater proportion of their resources to reproductive rather than somatic effort. In this study, the number of smaller males that developed crests as high or higher than those of larger males and also produced a similar number of spermatophores was low, suggesting that either only a few members of the population are both small and old or that, in the male smooth newt, utilisation of stored or current resources is more important in determining crest development and spermatophore production than the allocation of resources between current and future reproduction.

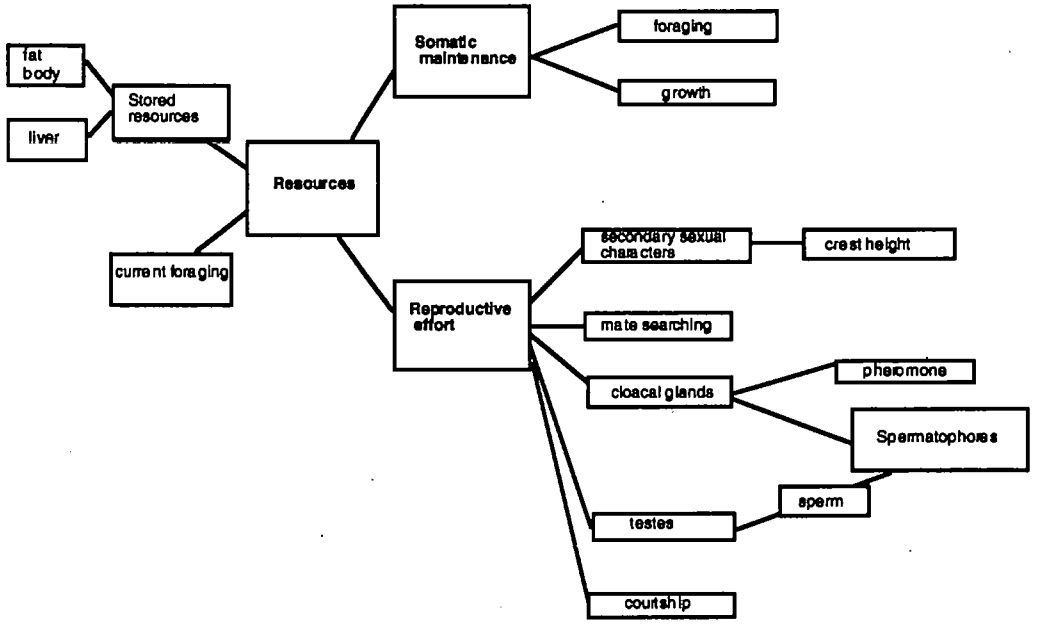


Fig 5.5. Allocation of resources between somatic maintenance and reproductive effort in the male smooth newt.

Kodric Brown & Brown (1984) describe this scenario as the 'truth in advertising' hypothesis. Honest signalling by a well-developed trait requires the allocation of limited resources to that trait, and diversion of such resources away from other components of somatic and reproductive effort. Therefore, the degree of development of the trait may reflect age, nutritional status, and resistance to predators and pathogens as well as body size. Thus sexual selection favours male traits which vary phenotypically between individuals, maintaining a positive correlation between degree of development of the traits and overall fitness.

5.6.2. Can larger males deposit spermatophores over a longer period during the breeding season than smaller males?

The duration of the deposition period is related to male body size, with respect to both snout-vent length and weight, when males are in relatively good condition. However, the best predictor of the length of time over which males deposited spermatophores is the maximum tail depth achieved by a male. The relationship between maximum tail depth and the duration of the deposition period is weaker than the relationship between maximum tail depth and total

spermatophore production, which may be a consequence of variation in the rate of spermatophore deposition among individuals. Some males with a low total spermatophore production failed to deposit spermatophores during every test, even though they had a shorter overall deposition period than males with a high total score. By not depositing spermatophores during every test, males with low total spermatophore scores increase the variance in the duration of the deposition period among individuals. The increased variation confounds the relationship between maximum tail depth and the duration of the deposition period, making the relationship weaker than the relationship between maximum tail depth and total spermatophore production.

The number of spermatophores produced during the breeding season is positively correlated with the length of the deposition period, so males with higher reproductive potential deposit a similar number of spermatophores per encounter but for longer in the season compared with males with lower reproductive potential. This finding may provide further evidence that the number of spermatophores deposited per encounter reflects the level of secretory products in the cloacal glands, especially as the sizes of the tubules in the cloacal glands have been shown to be correlated with the number of spermatophores deposited (Chapter 3). Alternatively, males may possess enough secretory products in the cloacal glands to continue spermatophore production, but may cease deposition because the costs of depositing further spermatophores to a single female outweigh the benefits.

Males with higher reproductive potential may be able to inseminate females over more days of the season. The maximum deposition period recorded (84 days, for one male) is a similar length to the maximum oviposition period recorded for a single female (74 days, Baker 1992a). Therefore, males with a good spermatophore supply are able potentially to inseminate females arriving after the period of early migration and to reinseminate females as they become responsive during the oviposition period. Consequently, males in good reproductive condition, possessing a good spermatophore supply and the ability to develop a high crest, are potentially able to realise higher reproductive success by endurance rivalry,

i.e. remaining in good reproductive condition longer than their rivals (Andersson 1994), as well as via scramble competition (Verrell & McCabe 1988), sexual interference (Verrell 1984a) and female choice (Green 1991; Hosie 1992).

Arak (1988) has proposed that the difference in body size between the two sexes of a species should be proportional to the difference between their reproductive selection gradients for size (Arnold & Wade 1984), a hypothesis supported in nine species of anurans. The similarity in body size between the sexes in smooth newts (Kalezic 1992) implies that the selection gradients for body size may be similar in the two sexes. Males prefer larger, more fecund females as mates (Verrell 1986c) and thus large male body size could be a consequence of genetic correlation with selection for large female body size (Lande 1980). However, the correlation between male body size and both spermatophore production and duration of the deposition period suggests that male body size is also under selection pressure from endurance rivalry and, perhaps, female choice. This suggestion is supported by females preferentially picking up spermatophores from males with larger crests (Hedlund 1990; Green 1991; Hosie 1992), and by a recent study which demonstrates that females mate sequentially with males possessing higher crests than their previous mate (Gabor & Halliday 1997). Therefore, if larger males can develop larger crests more easily than smaller males this may increase selection for larger male size.

Most previous studies (see Table 5.1), which have investigated reproductive success via short term mating capacity, have failed to establish a relationship between the number of spermatophores produced and male body size in the absence of male-male competition. An exception is the study in *T. cristatus* (Hedlund 1990), but this relationship only holds before the crest has developed fully. My findings are the first to demonstrate a relationship between two measures of seasonal reproductive potential, spermatophore production and duration of the deposition period, and both male body size and crest height, supporting the hypothesis that cross-sectional data may not accurately reflect potential reproductive success over a longer time scale. The finding that crest height is correlated with spermatophore production suggests that, in choosing to mate with a high crested male, a female is choosing 'good

genes' that will impart high fecundity and high vigour to her offspring. Therefore, this study also provides some evidence in support of the 'viability indicator' models for the acquisition of good genes.

5.7. Summary

Although seasonal spermatophore production was correlated with male body size (snout vent length and weight), maximum tail depth achieved by an individual male (an indicator of crest height) during the season was the best predictor of the number of spermatophores deposited. Tail depth and snout-vent length are correlated in smooth newts, suggesting that larger males may have an advantage over smaller males. However, there is much variation in the maximum tail height achieved, and some smaller males were able to develop high tails and crests and also to produce high numbers of spermatophores.

The number of spermatophores produced was correlated with the length of the deposition period, showing that males with higher reproductive potential may be able to inseminate a larger number of females over the course of the season. The maximum deposition period recorded was of similar length to the maximum oviposition period recorded for a single female. Therefore, males in good reproductive condition are potentially able to realise higher reproductive success by endurance rivalry as well as via scramble competition, sexual interference and female choice.

Seasonal spermatophore production was not related to body condition on migration, suggesting that spermatophore production may be related to body condition the previous year when males were undergoing spermatogenesis (Chapter 1). Tail depth is condition dependent and, therefore, may be partly reflecting foraging ability, and partly reflecting genotypic differences in utilisation of resources.

These results present further evidence that the dorsal crest of male smooth newts may be an honest indicator of viability and may be under selection pressure through both endurance rivalry and female choice.

5.8. Multiple mating during one reproductive episode

Most mating activity in smooth newts occurs at dusk (Griffiths 1985), suggesting that males may have a daily window of mating opportunity, which is too narrow to allow males to replenish their supplies of sperm accessory materials between matings (Verrell 1986a; Chapter 3). Thus male reproductive success may be limited by the level of secretory products in the cloacal glands if the opportunity to mate with several females, other than as a result of female interference during a single encounter (Waights 1996), arises within one evening. An early study by Verrell (1986a) demonstrated that a male courted by a strait-jacketed female, in the presence of multiple mates, is unable to deposit spermatophores to more than one female. However, Verrell noted that a strait-jacketed female may represent a 'super-responsive' mate, as a standardised courtship encounter results in male sexual exhaustion.

Several subsequent studies support Verrell's suggestion, as more spermatophores (up to nine) may be elicited by strait-jacketed females (Baker 1990a; Chapter 4 and section 5.3), than by 'freely courting' females (Halliday 1974; Hosie 1992). The discrepancy observed between the two types of courtship encounters may be due to the way in which the encounters are terminated. A standardised courtship encounter is designed to elicit spermatophores from a male until he fails to deposit spermatophores within a certain time, whereas during 'free' courtship encounters, the courtships are under female control. 'Free' courtship encounters are terminated by the females swimming away, which is the natural conclusion to many of the courtship encounters in the field (Verrell & McCabe 1988). Taken together, the findings in the laboratory studies suggest that males may be able to mate multiply when courtship encounters are under female control. However, males with fewer spermatophores may be less attractive to females (Halliday & Houston 1978), so females may decline to mate with recently depleted males.

Spermatophore production over the season (section 5.3), which may indicate sperm supply, and the size of the tubules that synthesise sperm accessory materials (Chapter 3)

are both related to male body size. Thus the ability to court and inseminate multiple females during a single evening, when males are unable to replenish their supplies of sperm and sperm accessory materials between matings, may be related to male body size and may lead to enhanced reproductive success for larger males.

5.9. Aims

This study aims to test the hypotheses that male smooth newts can mate multiply during a single reproductive episode, when the encounters are under female control, and that the ability to mate multiply is related to male body size. A single reproductive episode is defined as encompassing a series of courtship encounters occurring consecutively, within one hour. This time constraint prevents males replenishing the secretory products of the cloacal glands between encounters (Verrell 1986a; this study). The encounters were staged so that sexual interference by individuals of either sex was prevented.

Hypothesis: H_1 Males can mate multiply during a single reproductive episode when the encounters are under female control.

Prediction : *1* Males court and successfully transfer sperm to more than one female during a single reproductive episode.

Hypothesis: H_2 The ability to mate multiply during a single reproductive episode is dependent on body size.

Prediction : 2 Larger males mate with more females during a single reproductive episode than smaller males.

5.10. Methods

Twenty-one male smooth newts, representative of the range in body size, were collected, mid season in 1994, and maintained (as described in section 2.3) for one week, without access to females, to ensure that the cloacal glands were full. Observation tanks were divided in half with a glass sheet, and a once-mated female (shown to be receptive to remating, Chapter 6) was placed in each half and given ten minutes to settle. Each male was

randomly allocated to one of the pair of females and allowed to initiate courtship. All courtships were allowed to proceed until the female interrupted courtship by swimming away. If five attempts by the male to continue the encounter each resulted in the female swimming away, the encounter was considered to have been terminated. The number of spermatophores deposited by the male to this female was recorded (score 1).

Immediately following termination of this first courtship the glass screen was lifted to allow the male access to the second female and replaced, trapping the first female in her half of the tank to prevent her interferring in the second encounter. The courtship encounter was allowed to proceed as described for the first encounter. The number of spermatophores deposited by the male to the second female was recorded (score 2). Immediately following termination of this second encounter the male was approached by a strait-jacketed female to determine whether the male could deposit spermatophores to a third female. The number of spermatophores deposited by the male to the strait-jacketed female was recorded (score 3). Female newts need time to settle in an observation tank prior to mating, so a maximum of two 'freely courting females' could be used in this study because of the size of the observation tank, which is why additional spermatophores were elicited using a strait-jacketed female.

5.10.1. Morphometrics

The following morphometric measurements were taken (as described in Chapter 2, section 2.4.3): snout-vent length (svl), weight (wt) and tail depth (td).

5.11. Results

5.11.1. Multiple mating during a single reproductive episode

A significant number of males (18 out of 21) were able to inseminate more than one female during a single reproductive episode (n_1=18, n_2=3, Exact binomial test: p=0.0015, Fig 5.6). There was no difference between the number of males that were able to inseminate two females only and the number of males that still had sufficient spermatophores to be able to respond to the strait-jacketed female (n_1=11, n_2=7, Exact binomial test: p=0.481). Over

80% of the males tested (17 out of 21) were able to inseminate both 'freely courting' females (Fig 5.6). Almost 50% of these (7 out of 17) were able to put down further spermatophores in response to the strait-jacketed female. Only 19% of the males (4 out of 21) failed to deposit any spermatophores during the encounter with the second female. Each of these males deposited four spermatophores in response to the first female, which is the same as the total number of spermatophores deposited to two females by some males, although one male went on to deposit two further spermatophores in response to the strait-jacketed female.

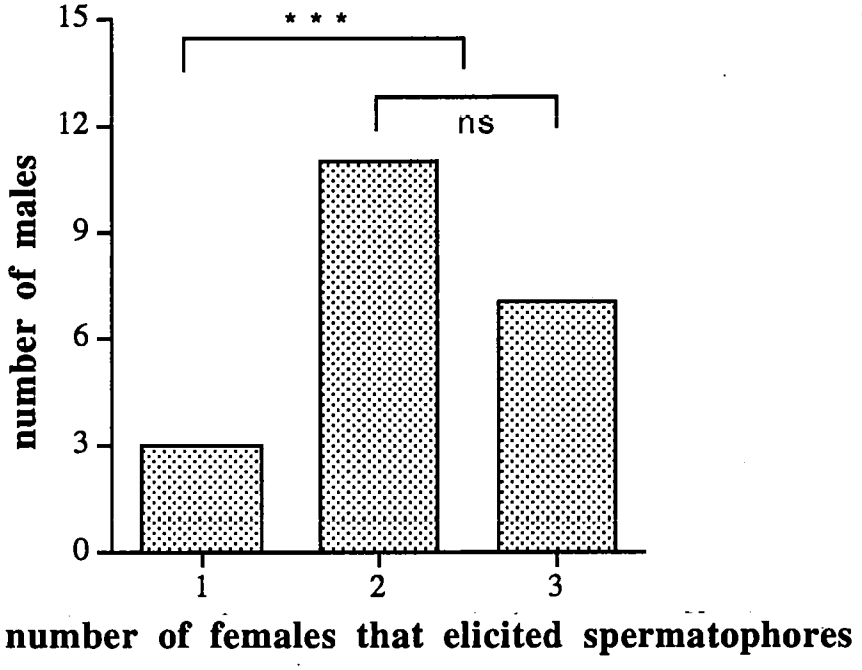


Fig 5.6 Frequency distribution of the number of males depositing spermatophores in response to one, two or three females during a single reproductive episode (Exact binomial tests: ***p=0.0015, ns=non significant)

Discriminant Analysis (Statistica), with number of mates as the grouping variable and snout-vent length [svl], and tail-depth [td] as the independent variables, revealed that the number of females that a male could inseminate during one reproductive episode was significantly related to tail depth but not to body size (Table 5.9). There was a positive trend for males able to mate multiply to be smaller than the males only able to mate once, but this may be an artefact due to the males with the lowest crests also being the largest males.

Two males have been excluded from this analysis, discussed in further detail in the next section, because they put down an exceptionally high number of spermatophores.

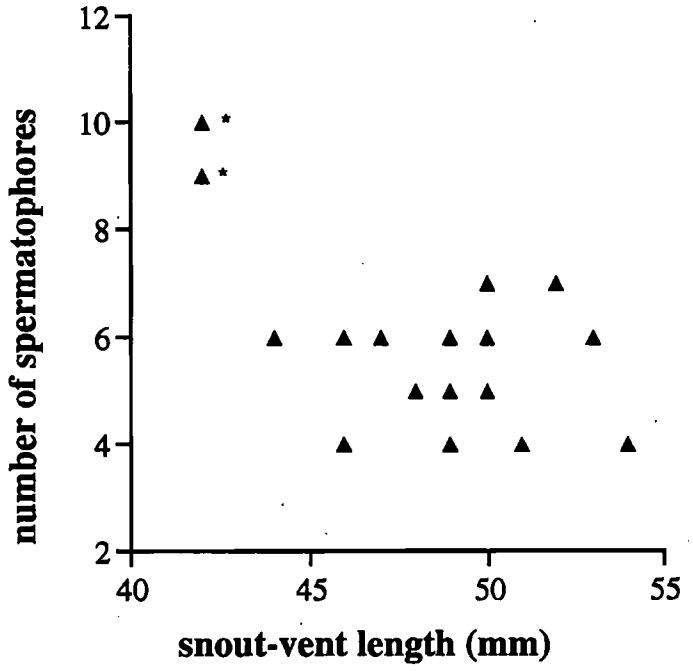
Table 5.9 Relationship between male body parameters and number of females that a male can mate with during a single reproductive episode (Discriminant analysis: **p<0.01; snout-vent length [svl]; tail height [td]).

	one mate	two mates	three mates	overall mean	partial lambda	p
n	3	11	5			
svl	51.3	48.6	49	48.4	0.68	0.06
td	11.8	12.3	13.7	12.6	0.51	**0.006****
correct classificat^{n}	66.6%	91.6%	75%	84%		

5.11.2. The effect of male body size on the total number of spermatophores deposited during a single reproductive episode

Partial correlation analysis revealed a negative correlation between the total number of spermatophores [spa] deposited and male body size (n=21, df=18, $r_{spa\ svl;td}$= -0.62, p<0.05). However, this relationship was no longer significant when the two outliers (shown in Fig 5.7 by asterisks) were removed (Table 5.10).

There was no relationship between tail depth and the total number of spermatophores deposited when all males were considered ($r_{spa\ td;svl}$=0.40, p>0.05), or when the two outliers were removed (Fig 5.7, Table 5.10). As the availability of additional spermatophores may influence the number of spermatophores elicited and subsequently picked up by the females, the two outlying males were excluded from the data set for all subsequent analysis.



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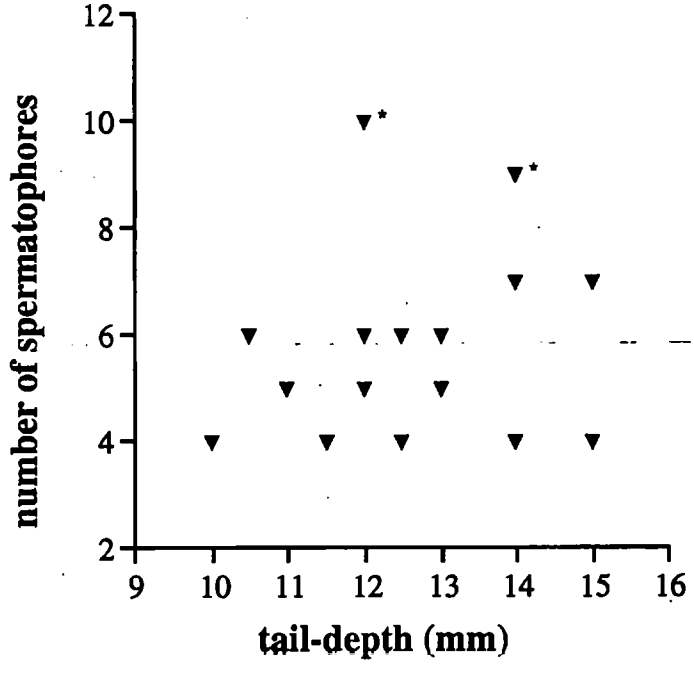


b)

Fig 5.7 Relationship between the number of spermatophores produced during a single reproductive episode and various parameters of male body size (Pearson product moment partial correlations: n=19, df=16, *outliers) a) male body size: $r_{spa\ svl;td}$= 0.16 p>0.05; b) tail depth: $r_{spa\ td;svl}$=0.33, p>0.05).

Table 5.10 Relationship between male body size [svl] and tail depth [td] and the total number of spermatophores deposited to all three females [total], to first and second female only [score^t], and picked up [pick-up^t] by both females during a single reproductive episode (Pearson product moment partial coefficients).

	total (n=19, df=16)	**score^t (n=15, df=12)**	**pick-up^t (n=15,df=12)**
svl	$r_{tot\ svl;td}$=0.16 p>0.05	$r_{scor\ svl;td}$=0.11 p>0.05	$r_{pu\ svl;td}$=0.12 p>0.05
td	$r_{tot\ td;svl}$=0.33 p>0.05	$r_{scor\ td;tsvl}$=-0.19 p>0.05	$r_{pu\ td;tsvl}$=-0.38 p>0.05

In four cases the male failed to deposit any spermatophores to the second female, so these cases were excluded from the data set for the analyses that follow. There was no relationship between male body size or tail depth and the number of spermatophores deposited in total (score^t) to the two 'freely courting' females. Neither was there any relationship between male body size and the number of spermatophores in total picked up [pick-up^t] by the two 'freely courting' females. An unexpected finding was the lack of correlation between the total number of spermatophores deposited and overall pick-up success (n=15, df =14, r=0.19, p>0.05).

These results indicate that the total number of spermatophores deposited during the reproductive episode, in response to both 'freely courting' females (score^t) or in response to all three females (total), was not related to either male body size or the height of the crest. Similarly, the total number of spermatophores picked up by the two females (pick-up^t) was not related to either male body size or crest height. The next section considers the two encounters separately, according to their ordinal positions.

5.11.3. The effect of male body size on the number of spermatophores deposited to each 'freely courting' female

There was no relationship between male body size (measured as snout-vent length) or tail depth and the number of spermatophores elicited (score) or picked up (pick-up) by either the first or second female (Table 5.11). Spermatophore score did not need to be partialled out of

the analysis between the male body size parameters and pick-up success because there was no relationship between spermatophore score and pick-up success for either female (n=15, df=14, female 1: r=0.03, p>0.05; female 2: r=0.38, p>0.05).

Table 5.11 Relationship between male body size [svl] and tail depth [td] and the number of spermatophores elicited [score] and picked up [pick up] by the first female (female1) and the second female (female2) to mate during a single reproductive episode (Pearson product moment partial coefficients: n=15, df=12).

	female 1		**female 2**	
	score	**pick-up**	**score**	**pick-up**
svl	$r_{svl\ scor;td}$=0.12 p>0.05	$r_{svl\ pu;td}$=0.13 p>0.05	$r_{svl\ scor;td}$=0.02 p>0.05	$r_{svl\ pu;td}$=-0.05 p>0.05
td	$r_{td\ scor;svl}$=-0.19 p>0.05	$r_{td\ pu;svl}$=0.34 p>0.05	$r_{td\ scor;svl}$=-0.03 p>0.05	$r_{td\ pu;svl}$=-0.32 p>0.32

5.11.4. The effect of order of courtship encounter on the number of spermatophores elicited and picked up by each 'freely courting' female

The number of spermatophores elicited by the first female to mate did not differ significantly from the number of spermatophores elicited by the second female to mate during a single reproductive episode. However, the number of spermatophores successfully picked up by the second female was significantly reduced compared with the number successfully picked up by the first female (Table 5.12).

Table 5.12 Comparison of effect of order of courtship encounter on the number of spermatophores elicited [score] and picked-up [pick-up] by the first female (female1) and the second female (female2) to mate during a single reproductive episode (Wilcoxon tests, corrected for ties: n=15, ***p<0.005, ns=non significant).

	female1	**female2**	**z**	**p**
SCORE				
range	2-4	2-4		
mean	2.73	2.40		
s.d	0.88	0.63		
median	3	2	-1.067	0.29ns
PICK-UP				
range	1-3	1-2		
mean	1.80	1.13		
s.d	0.56	0.63		
median	2	1	-2.803	**0.005*** **

During a male's first encounter, there was a trend for more females to initially become inseminated from the spermatophore deposited in the first ordinal position than from the spermatophore in the second ordinal position (Fig 5.8), although in both cases the females may continue to pick up spermatophores deposited in later positions. This trend was not observed during the male's second encounter (Fig 5.8).

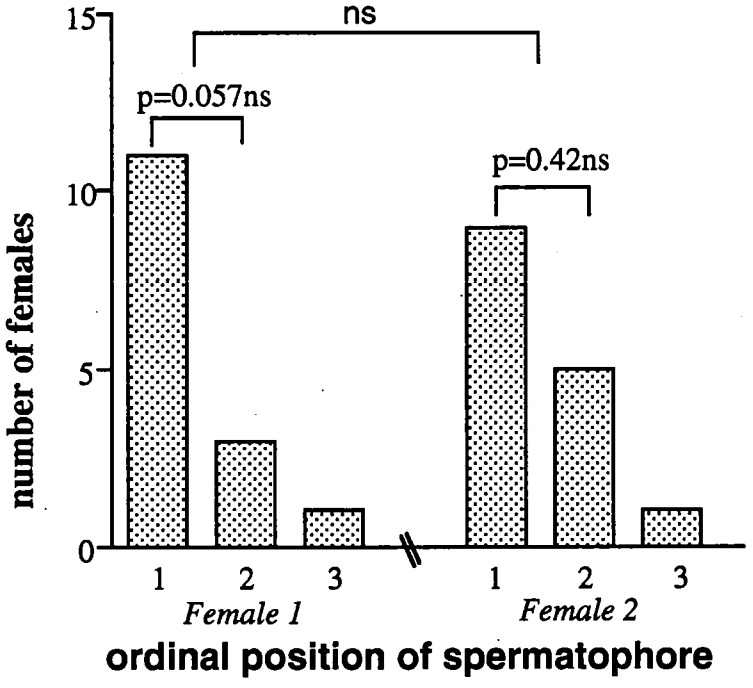


Fig 5.8 Comparison of ordinal position (either first or second) from which the first spermatophore is successfully picked up by the female during the first courtship encounter (female 1) and the second courtship encounter (female 2) with the same male (within females: exact binomial tests; between females: Fisher's exact test; ns=non significant).

Fisher's exact test (p=0.47) showed that there was no relationship between the ordinal position of the spermatophore which initially inseminated the female and the order in which the females encountered the male. In other words, the likelihood of a female picking up the first spermatophore deposited in an encounter was not related to whether the male had mated recently. This result was unexpected because males that have recently mated may be less effective at stimulating females than males which have replenished supplies of sperm accessory materials and pheromone, resulting in the females picking up spermatophores deposited later rather than earlier in an encounter.

The number of spermatophores elicited by the second female to mate was greater than the number of spermatophores deposited in response to the strait-jacketed female (Wilcoxon test, corrected for ties: n=15, z=-3.180, p=0.002). Only six males actually deposited any spermatophores in response to the strait-jacketed female (after depositing to both 'freely courting' females). However, when the males that failed to deposit any spermatophores are excluded from the analysis, there is still a negative trend (Wilcoxon test, corrected for ties: n=6, z=-1.8257, p=0.067), although non significant, between the number of spermatophores deposited in the two encounters. This lack of correlation may be due to the small sample size.

5.12. Discussion

5.12.1. Can males mate multiply during a single reproductive episode?

These results demonstrate that a substantial number of males were able to inseminate more than one female during a single reproductive episode. However, only 33% of the males were able to deposit spermatophores in response to a third (strait-jacketed) female. Taking into consideration that approximately 10% of males may fail to respond to strait-jacketed females (Halliday pers comm), over 50% of the males are unable to respond to a third female. As explained in Chapter 2, a strait-jacketed female mimics a highly responsive female. Consequently, the failure of the majority of males to respond to the strait-jacketed female, coupled with the trend for a reduced number of spermatophores to be deposited when males do respond, suggests that the males reached their mating capacity during this test. The implications of this finding on seasonal mating patterns are discussed in Chapter 8.

5.12.2. Is successful multiple mating during one reproductive episode dependent on male body size?

My findings suggest that the ability of individual males to mate multiply during one reproductive episode is dependent on their reproductive condition (which is reflected in their tail height), but not their absolute body size, even though the total number of spermatophores deposited during the episode is not related to either reproductive condition or body size. My

findings, together with other studies (Verrell 1986a; Baker 1990a; chapter 4) suggest that a strait-jacketed female, mimicking a highly responsive female, may encourage males to deposit more spermatophores in response to a single female than would occur naturally. Standardised courtship encounters, therefore, probably demonstrate the maximum potential spermatophore production of an individual at a given time, rather than give an indication of the number of spermatophores that an individual male would deposit in response to a single female. Consequently, although the study in the first part of this chapter may give a good indication of the total number of spermatophores an individual has available for deposition during a single reproductive season, it does not give a reliable indication of the number of mates that an individual can potentially inseminate. Similarly, this study has shown that the lack of relationship between spermatophore production and male reproductive condition (Baker 1990a; chapter 4), is masking a relationship between reproductive condition and the ability of males to mate multiply.

This study shows that the number of spermatophores deposited to two 'freely courting' females does not exceed the number deposited in response to a strait-jacketed female (chapter 4 and section 5.3). Thus courtship encounters may be under female control and multiple deposition and multiple insemination may occur as a result of the female's continued interest in the courtship. This finding concurs with Halliday's (1974; 1975) studies in which he found that successful sperm transfer early in an encounter does not alter the probability that the encounter will continue.

The similarity between the total number of spermatophores deposited in response to two 'freely courting females' and the number of spermatophores deposited in response to strait-jacketed females (chapter 4) also supports the rationale behind this study, that males are unable to replenish the secretions of the cloacal glands within one hour of mating. In the study in Chapter 3, 78 males were tested using a standardised courtship encounter; of these, four males deposited seven spermatophores, four males deposited eight spermatophores and one male deposited nine spermatophores in response to the strait-jacketed female, suggesting that the deposition of more than seven spermatophores during a single courtship encounter is

rare. Both males in this study that deposited a high number of spermatophores in total (9 and 10) were very small (snout-vent length 42 mm). Seasonal spermatophore production is related to male body size (section 5.3), so deposition of a high number of spermatophores coupled with their small body sizes suggests that these males had yet to utilise any of their seasonal supply of spermatophores. The high number of spermatophores each male was able to deposit may indicate the accumulation of cloacal gland secretions from the beginning of the season (over one month earlier).

5.12.3. Do the males transfer as much sperm to the second female compared with the first female?

During a single reproductive episode, males transfer fewer sperm masses to the second female, even though a similar number of spermatophores were deposited in response to each. Reduced sperm transfer during the second encounter suggests that males may be less effective at stimulating the second female. The female may be inadequately stimulated because the male's display rate decreases during the second encounter; males with fewer spermatophores available for deposition display at a lower rate (Halliday & Houston 1978). Alternatively, the males' dorsal glands may be becoming depleted and are releasing less pheromone. Females are known to orient towards males via pheromone cues (Malacarne & Vellano 1987) and a reduced pheromone cue may make spermatophore transfer less efficient. During female sexual interference, females are thought to be able to pick up spermatophores that are deposited in response to a rival female by using pheromone cues to achieve the correct orientation (Waights 1996). An alternative possibility is that females are able to distinguish between recently and non-recently mated males, which occurs in other species, e.g. butterflies (Rutowski 1979) and lemon tetra (Nakatsuru & Kramer 1982).

Consequently, highly proceptive females (terminology of Beach 1976), such as the once-mated females in this study, may be the only females willing to mate with recently mated males and suffer the cost of reduced sperm transfer. Sperm transfer is reduced both in terms of the number of spermatophores picked up and in the number of sperm contained within the

sperm masses of spermatophores deposited later in a series (Chapter 4). It may be fruitful to repeat this study using females that are ovipositing, to determine whether females are able to distinguish between recently and non-recently mated males.

5.13. Summary

The ability of males to mate multiply during a single receptive episode was related to their reproductive condition (measured as crest height), but not to their absolute body size (snout-vent length). However, crest height was not related to the total number of spermatophores deposited during the reproductive episode.

A similar number of spermatophores was elicited by each female, yet the first female to mate picked up more spermatophores than the second female to mate, suggesting that only highly receptive females may be motivated to mate with depleted males. It is possible that males able only to mate with one female were limited by spermatophore supply; however, the sample size was too small to test this statistically.

Chapter 6. Sperm utilisation in the female smooth newt

6.1 Multiple mating in females

A single mating has been considered sufficient to fertilise all of a female's current offspring (Parker 1970), suggesting that multiple mating by females would not increase their reproductive success (Halliday & Arnold 1987). Each mating may also incur costs to the female, such as the actual time and energy spent in courtship and copulation, increased risk of predation during mating and adverse effects of male seminal products. Male seminal products may transmit disease (Thrall *et al.* 1997), although there is no evidence of sexually transmitted diseases occurring in newts (Sever pers comm), or may reduce female longevity, e.g. in *Drosophila* (Chapman *et al.* 1995), but the effect of mating on female longevity has not been studied in urodeles. Despite these potential costs, multiple mating by females, resulting in multiple paternity of offspring, has been observed in many taxa in the wild, including, mammals (Birdstall & Nash 1973; Murie 1995); birds (Gibbs *et al.* 1990; Birkhead & Møller 1992); reptiles (Barry *et al.* 1992); insects (Thornhill & Alcock 1983); newts, e.g. *Triturus alpestris* (Rafinski 1981) and salamanders, e.g. *Desmognathus ochrophaeus* (Tilley & Hausman 1976; Labanick 1983).

Little is known regarding the mating patterns of individual female smooth newts or the paternity of their offspring in the wild. However, in the laboratory female smooth newts mate multiply, both prior to the onset of ovulation and during the long oviposition period

(Verrell 1984b; Hosie 1992), even though they may be multiply inseminated by a single male (Halliday 1976) or by two rival males (Verrell 1984a) during a single encounter.

Current hypotheses propose either that multiple mating is adaptive for females (Alcock *et al.* 1978; Halliday & Arnold 1987) or that it is a consequence of genetic correlation with selection for multiple mating in males (Halliday & Arnold 1987). This latter theory is controversial; several authors have questioned whether it is supported empirically (Sherman & Westneat 1988; Cheng & Siegel 1990; Gromko 1992). Arnold & Halliday (1992) have challenged the rigor of the empirical evidence used to dismiss the genetic correlation hypothesis and their theory is still considered to be a plausible mechanism by which multiple mating is selected (Reynolds 1996), although Birkhead (1995) concludes that this theory is effectively discounted.

Adaptive hypotheses to explain multiple mating in females are grouped according to whether they impart direct or genetic benefits to the female (Birkhead 1995; Reynolds 1996, Table 6.1). Some hypotheses are unlikely to be relevant in smooth newts, due to the absence of social groupings and parental care. Similarly, it is unlikely that the spermatophore provides nutrients for female newts because the spermatophore base is abandoned on the substrate and the females appear to remove the matrix of the sperm mass from their cloaca 15-30 min after mating (pers obs). However, female insects have been shown to digest sperm and use them as a nutrient source; proteins derived from sperm were incorporated into the ova that were subsequently laid by the females (Parker 1970; Sivinski 1984; Eberhard 1996). It is known that phagocytosis of sperm occurs in the spermathecae of some newts and salamanders (discussed under sperm storage in the next section), but whether this is to obtain nutrients has not been investigated. Thus the hypotheses most likely to explain multiple mating in female smooth newts concern acquisition of sperm, initiation of ovulation, sperm competition and mate choice.

Table 6.1 Adaptive hypotheses to explain multiple mating in females.

Direct Benefits

1) to insure an adequate sperm supply (Gromko *et al.* 1984a; Levitan & Petersen 1995).

2) to insure against defective sperm (Taylor 1967) or the first mate being infertile (Gibson & Jewell 1982).

3) to induce ovulation and oviposition (Baumann 1974), which may enable females to fine-tune reproduction to occur when sperm are present in the storage organs.

4) to minimise the physiological costs of sperm maintenance during storage (Thornhill & Alcock 1983). Storage for long periods may require females to produce secretions that nourish the sperm to retain sperm viability, in which case it is less costly to maintain a few sperm and to replenish supplies than to maintain large numbers of sperm for a longer period.

5) to acquire nutrients in the sperm or accessory materials (Sakaluk & Cade 1980) that are either incorporated into the ova or utilised by the females.

6) to reduce sexual harassment (Svärd & Wiklund 1986). Mating may be less costly for females than avoidance of mating.

7) to reduce disruption in a social group (Halliday 1980).

8) to secure paternal care (Davies 1985). Females may mate multiply with their partner during the breeding season to ensure that he contributes to the care of the offspring.

Genetic benefits

9) to acquire 'good genes' through sequential mate choice (Halliday 1983). Unmated females mate non-selectively at first, to ensure fertilisation of the clutch, and then remate only with males that are superior to their previous mate, e.g. with males possessing higher dorsal crests (Gabor & Halliday 1997).

10) to ensure male offspring have high fertilication ability as a result of inter-ejaculate competition for fertilisation, termed sperm sexual selection (Harvey & Bennett 1985; Keller & Reeve 1995).

11) to reduce meiotic drive, also termed segregation distortion (Haig & Bergstrom 1995). Gametes that possess the driving allele and gain a competitive advantage within their own ejaculate may reduce the competitiveness of their ejaculate against the ejaculates of other males. Any reduction in the ability to combat sperm competition would be disadvantageous to the sons of females. Multiple mating in females will favour competition between the ejaculates of different males, rather than within a single ejaculate, thus reducing the probability that the females' eggs are fertilised by gametes possessing the driving allele and reducing the probability that her sons' ejaculates will be disadvantaged in sperm competition.

12) to increase viability of offspring via genetic diversity or 'better genes' (Madsen *et al.* 1992). The occurrence of meiotic drive in one haploid genotype may be balanced by a viability advantage of the other haploid genotypes. Multiple mating in females will increase the probability of her offspring being fertilised by non-driving genotypes and as the non-driving genotypes are more viable, the viability of her offspring will increase in comparison to the offspring of a female than mates singly (Haig & Bergstrom 1995).

6.1.1. Sperm transfer by male smooth newts

Male smooth newts arrive at a pond with a finite supply of sperm, which must be partitioned judiciously between the sperm masses of individual spermatophores. The findings in chapter 4 suggest that each spermatophore contains sufficient sperm (38 000-148 000) to fertilise an entire clutch (up to 600 eggs). However, this conclusion takes no account of the fate of the sperm within the female reproductive tract. In many taxa, sperm are differentially selected in the female tract and few of the sperm transferred during mating reach the storage organs or the site of fertilisation (Birkhead *et al.* 1993). Thus it is plausible that female smooth newts may become multiply inseminated during a single encounter or may mate multiply before oviposition to acquire sufficient sperm to fertilise their full clutch.

6.1.2. Sperm Storage

The way in which sperm are stored in the female reproductive tract may influence the pattern of mating exhibited by a female. Female smooth newts store sperm in spermathecae, which consist of numerous, simple glands formed by invaginations of the cloacal epithelium (Boisseau & Joly 1975; Verrell & Sever 1988; Sever 1994b) but the precise function of the secretory products of the glands is unknown. Dent (1970) and Boisseau and Joly (1975) proposed that they may nourish the sperm, but more recently, Sever and Kloepfer (1993) proposed that the secretions provide the chemical and osmotic environment for sperm quiescence, which is observed in sperm stored in the spermathecae (Hardy & Dent 1986b).

Sperm storage may be costly for females; only live sperm enter the spermathecae of female *Notophthalmus viridescens*, which are thought to be too small to contain all the sperm from a single spermatophore (Hardy & Dent 1986a). It is not yet known whether sperm transport and storage are similar in smooth newts.

Even if the number of sperm reaching the female's spermathecae is sufficient to fertilise her entire clutch, the sperm may not be retained in the spermathecae long enough for this to be realised. Sperm stored in the spermathecae of female urodeles of the superfamily

Salamandroidea may be degraded by the spermathecal epithelium, which is actively spermiophagic during sperm storage, e.g. in *Ambystoma opacum* (Sever & Kloepfer 1993), in *Notophthalmus viridescens* (Dent 1970), and in *Salamandrina terdigitata* (Brizzi *et al.* 1995b) or they may be phagocytosed by leucocytes (Sever 1991; 1992; Sever & Brunette 1993; Sever & Kloepfer 1993; Brizzi *et al.* 1995b). Verrell and Sever (1988) did not find close associations between sperm and the spermathecal epithelium in *T. vulgaris*, although Wahlert (1953, cited in Verrell & Sever 1988) found sperm embedded in the epithelial cells. In other species in which sperm are also stored in a spermatheca, sperm has been shown to lose viability with time in storage, e.g. in *Drosophila* (Hiraizumi & Watanebe 1969). If this occurs in smooth newts, phagocytosis may be enabling females to remove non-viable sperm from the spermathecae.

Thus constraints on sperm storage may result in females needing to remate during oviposition to gain sufficient sperm to fertilise their full clutch. Verrell (1984b) and Hosie (1992) found that females mate infrequently during oviposition, but the cues that induce receptivity in ovipositing female newts are unknown. In insects, diminishing supplies of sperm in the spermatheca may induce sexual receptivity in females (Nakagawa *et al.* 1971), via relaxation of stretch receptors in the spermathecal wall (Taylor 1967). At present, it is not known whether this type of feed-back system occurs in any species of urodele.

6.1.3. Initiation of ovulation

Only two studies, in *Ambystoma mexicanum* (Humphrey 1977) and in *Taricha granulosa* (Moore *et al.* 1979), have investigated the onset of ovulation in urodeles, and both studies found that females require courtship display and insemination to induce ovulation. Non-inseminated female *T. vulgaris* do not lay eggs or only lay a few infertile eggs (Pecio 1992; pers obs), which suggests that they also require courtship display and insemination to induce ovulation. Courtship induces changes in the GnRH (gonadotrophin-releasing hormone) concentrations in the brain of female *Taricha granulosa* (levels are high when courtship is initiated but decrease by the time of sperm transfer) which may interact with the female's

reproductive system to initiate oviposition (Propper & Moore 1991), but to date nothing is known concerning hormone levels in smooth newts.

6.1.4. Offspring viability

Selection may act on females to mate multiply to increase the viability of their offspring by increasing their genetic diversity, although to date few studies support this theory empirically. Multiple paternity in adders has been shown to increase the viability of offspring in one population (Madsen *et al.* 1992; Olsson *et al.* 1994), although this effect was not observed in another population (Capula & Luiselli 1994). Madsen *et al.* (1992) proposed that the increase in offspring viability was due to the ova being fertilised by sperm from 'better-males' via sperm competition, rather than due to increased genetic diversity resulting from polypaternity. At present, there is no conclusive evidence that sperm (the haploid stage) which are more successful in fertilisation also produce more viable offspring (the diploid stage). Parker (1992) postulated several alternative mechanisms that could also explain these results, e.g. reduced storage time of sperm and therefore less effect of sperm ageing or fitter males transferring more sperm than less fit males. A recent study in smooth newts has shown that female smooth newts mate sequentially with males with larger crests (Gabor & Halliday 1997), suggesting that female smooth newts may also remate to increase the viability of their offspring.

As described in Chapter 1, oviposition is time-consuming and female smooth newts may take up to 70 days to lay a full clutch (100-600 eggs, depending on their body size, Baker 1992a). Therefore, long-term storage of sperm (see discussion of sperm storage above) or remating to gain supplies of fresh sperm, due to sperm deterioration during storage, may be important in this species. Female newts may mate multiply at the beginning of the season (Hosie 1992) to acquire sufficient sperm to fertilise their full clutches, e.g. if one sperm mass does not contain sufficient sperm, and, possibly, to invoke sperm competition. Thus they may only remate during the oviposition period if superior mates are available. Alternatively, females may remate during the oviposition period to replenish their supplies of

sperm, due to either limited sperm storage capacity or to loss of viability of the sperm during storage.

6.2 Aims

This study aims to elucidate the role of sperm transfer, sperm storage, and courtship display in multiple mating by female smooth newts. The effect of courtship display and differential sperm supply on the onset of oviposition, clutch size, duration of the oviposition period and egg viability will be investigated. From the preceding theory (discussed in chapter 4 and the introduction to this chapter), several hypotheses can be postulated which are outlined below (Table 6.2).

Table 6.2 Hypotheses and predictions concerning multiple insemination and multiple mating by females.

Female newts become multiply inseminated during a single encounter:-	
Hypothesis: 1 to acquire sufficient sperm to fertilise an entire clutch because one sperm mass contains insufficient sperm.	*prediction: 1* females will be unable to lay an entire clutch after transfer of one sperm mass.
Female newts mate multiply prior to oviposition:-	
Hypothesis: 2 to acquire sufficient sperm to fertilise an entire clutch, e.g. because a single male transfers insufficient sperm to fertilise an entire clutch.	*prediction: 2* females will be unable to lay an entire clutch after transfer of more than one sperm mass from a single male.
Hypothesis: 3 to acquire 'better' genes by exercising mate choice.	*prediction: 3* offspring of females mated twice will have higher viability than offspring of females only mated once.
Hypothesis: 4 to initiate oviposition through exposure to male courtship display.	*prediction: 4* females only exposed to one courtship encounter may fail to oviposit.
Female newts mate multiply during the oviposition period:-	
Hypothesis: 5 to replenish sperm supplies because storage capacity is limited.	*prediction: 5* females will be unable to lay an entire clutch from a single insemination.
Hypothesis: 6 to increase the viability of their offspring by acquiring fresh sperm because sperm deteriorates during storage.	*prediction: 6* the offspring of remated females will exhibit higher viability than offspring of females mated only prior to oviposition.
Hypothesis: 7 to increase the viability of their offspring via 'better' genes.	*prediction: 7* the offspring of remated females will exhibit higher viability than offspring of females mated only prior to oviposition.

These hypotheses are not all mutually exclusive. For instance, it may be difficult to distinguish between multiple mating during oviposition to acquire fresh sperm and multiple mating to acquire 'better genes', unless females that are prevented from remating fail to deposit their full clutch. Otherwise these two hypotheses can only be distinguished if DNA fingerprinting is carried out. However, multiple insemination and multiple mating may be responses to several selection pressures in males and females, which lead to patterns of spermatophore production, sperm transfer and sperm utilisation that enable each sex to exploit the other sex for their own benefit. Thus it is feasible that females remate to acquire fresh sperm and utilise this mating opportunity to exercise mate choice, or that males deposit multiple spermatophores to ensure insemination of each female but females exploit the transfer of 'excess sperm' by digesting them.

6.3 Methods

6.3.1. Collection of newts

Females were collected on land in March 1992 and March 1993 during their spring migrations to a breeding pond, and males in breeding condition were collected by hand-netting from another pond in Milton Keynes. The sexes were segregated and maintained as described previously (Chapter 2).

6.3.2. Experimental design

The experimental design is complex as this study aims to investigate the role of both the male and the female in sperm transfer and utilisation (Fig 6.1).

Initial insemination

This part of the experiment was designed to answer the following questions:-

a) can females lay an entire clutch from one spermatophore or do they need to be multiply inseminated by one male or to mate multiply to ensure adequate transfer of sperm?

b) do females require exposure to courtship display from additional males, without sperm transfer, to induce ovulation?

Courtship encounters were staged by making random pairings of male and female newts. Two unmated females were placed into each observation aquarium, 45x60x30 cm high, filled to a depth of 23 cm with water and kept at a natural photoperiod and temperature (approximately 1°C above that of the holding aquaria). Two males, selected at random from a stock tank and shown to be sexually active by pre-testing (described in chapter 2, section 2.4.6) were placed with each pair of females. The newts were given one hour to initiate courtship, after which time observations were terminated for unsuccessful pairings. All encounters initiated were followed and the females (25 in 1992 and 38 in 1993) were allocated, at random, to one of the following insemination regimes:-

a) females picked up just one spermatophore from one male (1 spa).

b) females picked up more than one spermatophore from one male (>1spa).

c) females picked up one spermatophore from each of two different males, with a two day interval between inseminations (2 males).

d) females picked up one spermatophore from one male. On three subsequent occasions, at two-day intervals, the females were courted during a single encounter (each involving a novel male). Each encounter was terminated at first spermatophore deposition and re-insemination prevented (1spa +ct).

e) females picked up more than one spermatophore from one male. Exposure to further courtship as above (>1spa +ct).

Three weeks post-insemination, the number of females in each group that had begun to oviposit was recorded. To establish whether failing to oviposit was a response to the treatment, each female that had failed to begin ovipositing was paired with a novel male. Each female was allowed to respond to courtship display until she had elicited deposition of a spermatophore from the male, but reinsemination was prevented.

Reinsemination

This part of the experiment was designed to determine whether females require fresh supplies of sperm during the breeding season to enable them to lay their entire clutch or to increase the viability of their offspring. Females that had commenced ovipositing were selected at random from the five initial insemination groups (described above) and allocated to one of two groups. The groups, matched for snout-vent length (SVL), were:

i) inseminated pre-oviposition only, mean SVL=46.8, s.d=2.91, n=9;

ii) inseminated pre-oviposition and during oviposition , mean SVL=47.7, s.d=3.11, n=8.

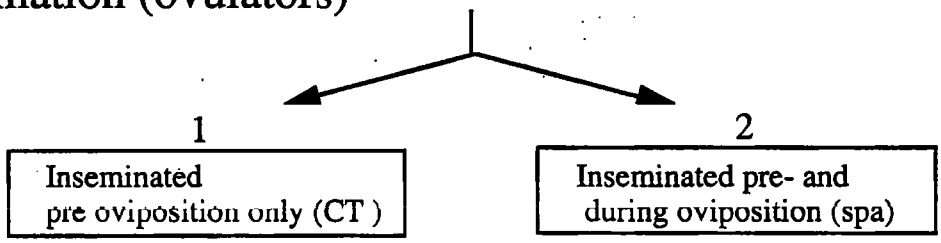
Initial Insemination

Encounter — Treatment

	a	b	c	d	e
1	1 spa	>1 spa	1 spa	1 spa	>1 spa
2			1 spa	CT	CT
3				CT	CT
4				CT	CT

Egg scores

Re-insemination (ovulators)



Encounter

5	CT	spa	CT	spa	CT	spa	CT	spa	CT	spa

Egg scores

Fig 6.1 Experimental design ('1 spa'=transfer of one spermatophore, '>1spa'=transfer of more than one spermatophore, CT=courtship without sperm transfer).

One female escaped and one female only oviposited for one day, so they were not included in this stage.

Insemination pre-oviposition only

Females in this group were not reinseminated during oviposition. To establish whether cessation of oviposition was a response to diminishing sperm supplies, each female was tested for receptivity to mating as described above and reinsemination was prevented.

Insemination pre-oviposition and during oviposition

Each female in this group was paired with a novel male and allowed to court until successful transfer of one or two sperm masses occurred.

6.3.3. Collection and maintenance of eggs

After insemination as outlined above, each female was housed outdoors in a separate tank (30x60x38 cm high), containing a refuge and artificial weed (as described by Baker 1992a). Female newts lay each egg individually and wrap it in a fringe of weed. Every one or two days during the season the eggs laid by each female were counted and removed by cutting the fringes off the strip. Two eggs, still wrapped in plastic, were placed into each individual well, containing 4.5 ml aged tap water, of 25-well petri boxes. The plastic boxes were covered with loose fitting lids to reduce evaporation, and the water level topped up as necessary. The eggs were maintained in an unheated outdoor shed until hatching and scored for viability. The experiment was concluded when the females ceased to lay eggs and began to show morphological changes commensurate with returning to the terrestrial phase.

6.4 Results

This study was carried out over two breeding seasons. A comparison of the proportion of females that had begun to oviposit during 1992 and 1993 revealed that there was no seasonal effect on the onset of oviposition (Fisher's exact tests: group a, $p_{5,8}$=1.70 ns; group b, $p_{4,4}$=2.00 ns; group c, $p_{6,9}$=0.57 ns; group d, $p_{5,10}$=1.14 ns; group e, $p_{5,7}$=1.38 ns). Therefore, the data for the two years were combined.

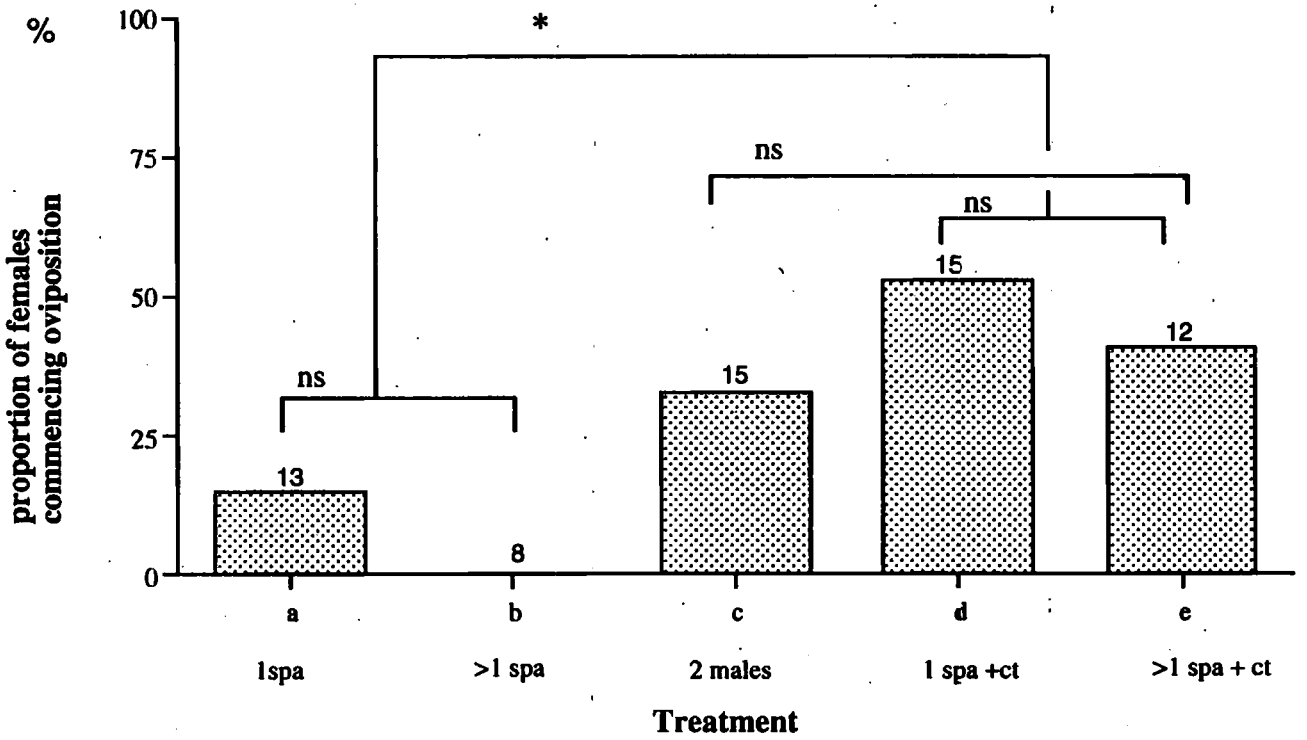


Fig 6.2 Effect of number of spermatophores picked up by the female, number of mates and further exposure to male courtship display on commencement of oviposition (number above column=number of females inseminated in that treatment group, 1spa=one spermatophore picked up by the female, >1 spa=more than one spermatophore picked up by the female, 2males=one spermatophore picked up by the female from each of two males,1 spa +ct=as 1spa plus further exposure to courtship, >1 spa +ct=as >1 spa plus further exposure to courtship. G-tests: *p<0.01, ns=non significant)

6.4.1. Onset of oviposition

The proportion of females that started to oviposit after single or multiple insemination by one male was low (10%, Fig 6.2). However, further exposure to male courtship display from novel males, post insemination by a single male, had a significant effect on the onset of oviposition (G-test: groups a and b combined versus groups d and e combined; n=48 g=9.0, $p<0.01$, Fig 6.2). The increase in the proportion of females beginning to oviposit was independent of the number of spermatophores picked up during the encounter (G-test: groups d and e; n=27, g=0.36 ns, Fig 6.2), and the number of males (2 or 1) that contributed the sperm, provided that the females inseminated by a single male received further courtship display from novel males (G-test: groups c and e, n=27, g=0.198 ns, Fig 6.2). Three weeks post-insemination, all females that had failed to commence oviposition elicited courtship display and spermatophore deposition from males during either the first or second encounter with a male, indicating that they were in reproductive condition and receptive to remating.

6.4.2. The effect of timing of insemination on the oviposition period

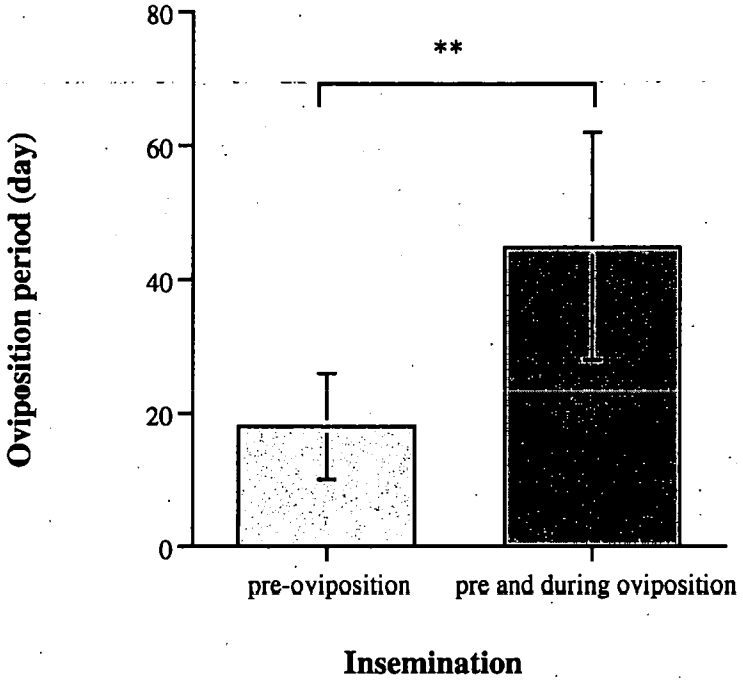


Fig 6.3. Effect of the timing of insemination a) pre-oviposition only and b) both pre- and during oviposition, on duration of the oviposition period (Mann-Whitney U-test: $n_1=9$, $n_2=8$, u=7.0, p=0.004**; error bars=s.d.).

Females inseminated only before the onset of oviposition exhibited a significantly shorter oviposition period than females that were reinseminated during the oviposition period (Fig.6.3).

6.4.3. The effect of timing of insemination on clutch size and egg viability

Females inseminated before the onset of oviposition laid a significantly smaller clutch (Fig. 6.4) containing a significantly higher proportion of non-viable eggs (G-test: n=2,965, g=89.9, p<0.001; Fig. 6.5) than females that were reinseminated during the oviposition period.

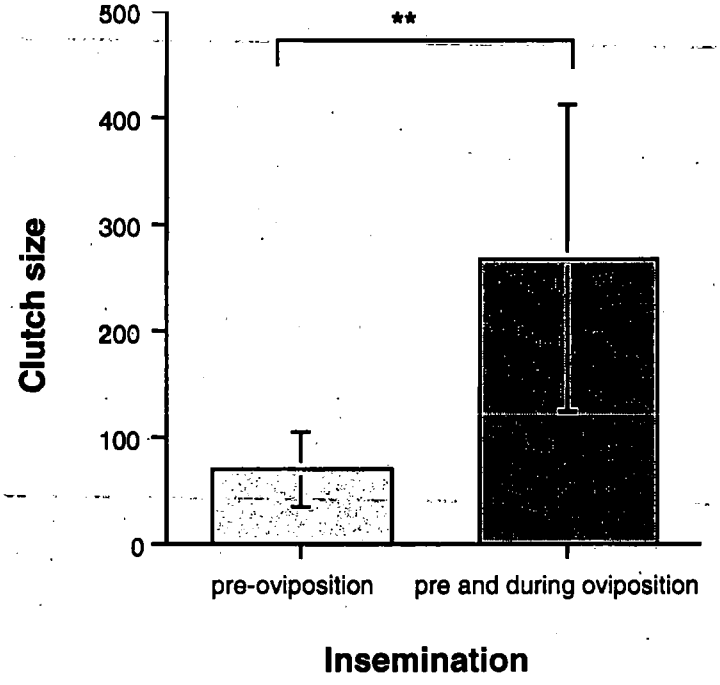


Fig 6.4. Effect of the timing of insemination a) pre-oviposition only and b) both pre and during oviposition on clutch size (Mann-Whitney U-test: $n_1=9$, $n_2=8$, u=1.5, p= 0.002**; error bars=s.d.).

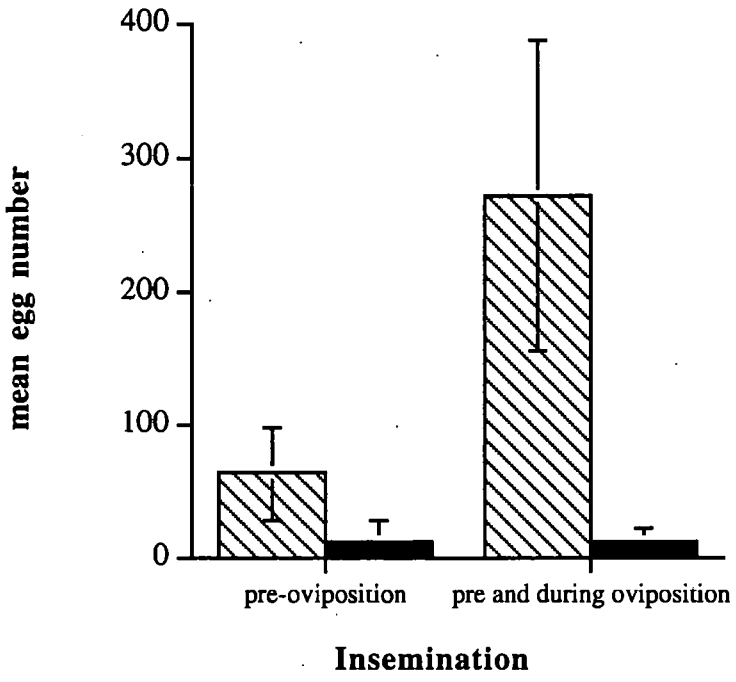


Fig 6.5. Effect of timing of insemination a) pre-oviposition only (n=9) and b) both pre-oviposition and during oviposition (n=8) on egg viability [shaded column=viable eggs, solid column=non-viable eggs, error bars=s.d.].

If the number of sperm stored in the spermathecae decreases with time or the stored sperm become less viable with time, then the viability of eggs laid from pre-ovipositional insemination would be expected to decrease during the oviposition period compared with eggs laid by females reinseminated during oviposition. To investigate this effect, each female's oviposition period was divided in half (by days) and the proportion of viable eggs laid early in the oviposition period was compared with the proportion of viable eggs laid in the later part of the oviposition period. In females only inseminated before the onset of oviposition, the viability of eggs laid early in the oviposition period was significantly higher than the viability of eggs laid late in the oviposition period. However, no difference was seen in the viability of eggs laid during these two periods in females that were reinseminated during the oviposition period (Fig 6.6).

Irrespective of timing of insemination, the number of eggs laid by a female during the early part of her oviposition period was higher, though not significantly, compared with the number of eggs laid during the later part of her oviposition period (Mann-Whitney U-test: $n_1=17$, $n_2=17$, $u=91.5$, $p=0.07$).

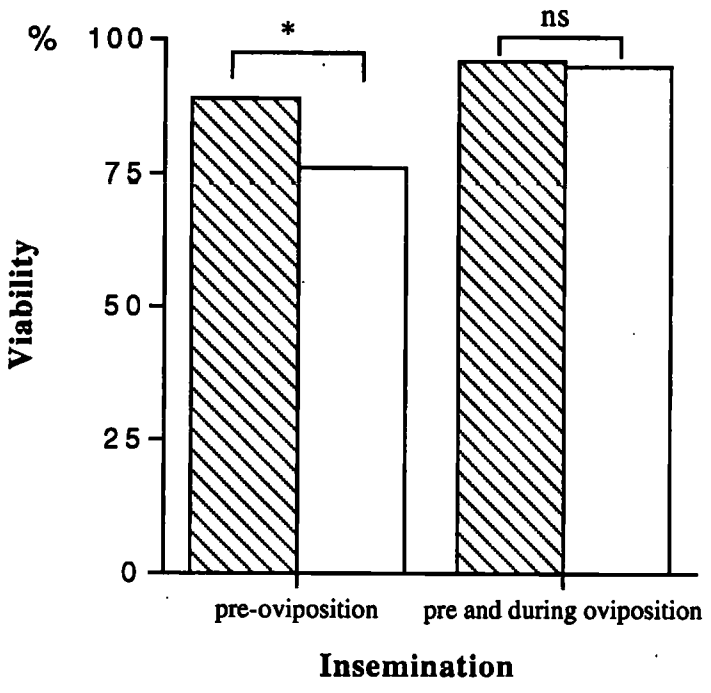


Fig 6.6. Comparison of viability of eggs laid early in the oviposition period [shaded with lines] and eggs laid later in the oviposition period for insemination a) pre-oviposition only (n=9 females, G-test: n=682, g=18.86, $p<0.05^{*}$) and b) both pre and during oviposition (n=8 females, G-test: n=2,283, g=0.02, p>0.05; ns=non significant).

6.5 Discussion

6.5.1. Onset of oviposition

Females in this study did not lay fertilised eggs until they were inseminated, confirming an earlier study by Pecio (1992). Female newts migrating to ponds in spring have been shown to possess a few sperm from the previous season in their spermathecae, e.g. in *T. vulgaris* (Verrell & Sever 1988) and in *Notophthalmus viridescens* (Massey 1990), but these sperm do not appear to be used to fertilise a second season's clutch. Female *N. viridescens* kept in captivity for a year, and hormonally induced to oviposit, laid infertile eggs and after oviposition their spermathecae did not contain sperm, suggesting that sperm from the previous season were not retained (Sever *et al.* 1996). However, it is also possible that the few sperm retained from the previous season were expelled from the spermathecae during oviposition. If this did occur, it is not clear whether the eggs were infertile due to the sperm being non-viable, or due to lack of sufficient sperm numbers for fertilisation.

This present study suggests that female smooth newts require exposure to male courtship display as well as insemination to induce oviposition. Unmated females, kept outdoors and never exposed to aquatic males, occasionally 'drop' a few unfertilised eggs on the base of the tank but have not been observed to lay unfertilised eggs on the weed (Hosie pers comm, pers obs). These observations appear to contrast with Pecio (1992), who noted that 12 out of 37 females laid a small number of infertile eggs prior to insemination. However, 11 of the 12 females laying unfertilised eggs had been courted previously by males, although not inseminated. My findings suggest that these females laid unfertilised eggs in response to male courtship display, which induced ovulation. The low number of eggs laid by these females is probably a result of the lack of other appropriate stimulation due to the absence of sperm in the spermathecae, which has been shown to occur in other urodeles. For example, even when female *Eurycea* are injected with hormones that induce ovulation, they fail to oviposit unless they have been mated previously (Sever pers comm).

Induced ovulation may have evolved as a consequence of smooth newts breeding in unpredictable environments, such as temporary ponds that may not fill with water every year. Oogenesis takes many weeks to complete (discussed in Chapter 1), so females may not be able to 'switch on' oogenesis quickly in response to environmental cues. Consequently, oogenesis takes place during the autumn and winter of the previous year and females use other environmental and physiological cues, including male courtship, to fine-tune ovulation and oviposition to ensure that their offspring have the best chance of survival. Male pheromones released during courtship may trigger hormonal changes that induce oviposition via the hypothalamic-pituitary-gonadal axis. Such a mechanism may be comparable to the induction of ovulation in other species, which occurs via a variety of mechanisms, including transfer of active factors during insemination, e.g. paragonial substance, PS-2 in *Drosophila funibris* (Baumann 1974) and behavioural stimulation, e.g. vocalisation in female doves (Cheng 1992) and multiple intromissions in rodents (Wilson *et al.* 1965; Lanier *et al.* 1975; Dewsbury 1984).

6.5.2. Multiple insemination during a single encounter

My study has shown that transfer of one sperm mass, or of multiple sperm masses from one male during a single encounter, is insufficient to fertilise a full clutch. These findings agree with a study by Verrell (1986c), which found that the number of eggs laid following insemination by one spermatophore is low, but contrasts with a laboratory study by Pecio (1992) in which she concluded that insemination by one spermatophore may be sufficient to fertilise a full clutch. The answer to this apparent dichotomy may lie in comparison of the temperatures during oviposition. The temperatures of Verrell's study (10°C) and my study (5-12°C) were comparable, whereas Pecio's study was carried out in a laboratory, which suggests that the temperature have may been higher. Hosie (1992) has shown that the number of eggs laid per day is temperature dependent. In a laboratory, the temperature may be sufficiently high to allow females to lay all their eggs relatively quickly, and the effects of long term sperm storage may not be so marked. This explanation is further supported by differences in the lag time between insemination and onset of oviposition under the two experimental conditions. Outdoors, onset of oviposition occurs within 6-10 days post insemination (Hosie 1992; this study) whereas in Pecio's laboratory study, onset of oviposition occurred mainly within 3-6 days post insemination. It should also be noted that female newts in Pecio's study typically lay up to 200 eggs whereas newts in the UK can lay up to 600 eggs (Baker 1992a), which may also lead to a different interpretation of the results.

Multiple insemination may be adaptive, e.g. to ensure that the female's spermathecae are saturated with sperm, or may occur as a consequence of female physiology. It is possible that females become multiply inseminated because they are unaware of successful pick-up of a sperm mass. In *Notophthalmus viridescens*, sperm from a sperm mass may continue to enter the spermathecal tubules for several hours post insemination (Hardy & Dent 1986a), which is also likely occur in smooth newts. If the feedback mechanism to prevent remating requires the presence of sperm in the spermathecae, this will not act sufficiently quickly to

prevent multiple insemination by a male during a single encounter or by several males during an encounter involving male sexual interference.

6.5.3. Temporal acquisition of sperm

As discussed previously, female smooth newts mate multiply both pre-oviposition and during the long oviposition period. Multiple mating prior to the onset of oviposition is consistent with several adaptive hypotheses, namely: to induce ovulation, to insure against the first mating being with an infertile male or as a consequence of female choice. This study has shown that females mate multiply to induce ovulation, but this does not preclude the other hypotheses. Hardy and Dent (1986a) have shown that only live sperm enter the spermatheca of female *Notophthalmus viridescens*. Thus, if female unresponsiveness to mating is related to the presence of sperm in the spermathecae, a female mated to an infertile male will not lose responsiveness, but will quickly seek out another male. However, infertile matings may be rare in smooth newts as none of the sperm masses tested in this thesis were devoid of sperm, which included the first sperm mass deposited by each of ten males at the start of the season. Females may also use the need for exposure to courtship display to mate selectively. Females may remate to insure that their offspring are fathered by genetically superior males, via either the mechanism of sperm precedence which allows the second male to mate to fertilise the majority of the eggs or by choosing to fertilise the eggs with sperm from the best male, but to date evidence for this is scarce. Last male precedence occurs in *T. alpestris* (Rafinski 1981) although total mixing and first male precedence also occur. Recent work by Rafinski (pers comm) has shown that sperm precedence may be determined by the time interval between the two matings (discussed more fully in chapter 4), but little is known regarding sperm competition in smooth newts.

Females inseminated only before the onset of ovulation laid smaller clutches than females reinseminated during oviposition, suggesting that female smooth newts remate after longer intervals to replenish sperm supplies. The spermathecae of female *Notophthalmus viridescens*, the only newt species studied to date, are estimated to be too small to contain all

the sperm from a single spermatophore (Hardy & Dent 1986a). My findings suggest that the storage capacity of the spermathecae of female smooth newts is also limited. Remating to replenish sperm supplies, as a consequence of limited storage capacity, has been shown to occur in female *Drosophila melanogaster*, both in the laboratory (Gromko *et al.* 1984b) and in the field (Gromko & Markow 1993), and is thought to be a strategy to minimise the costs of sperm storage. As discussed earlier, the secretory products in the spermathecae of female newts may nourish stored sperm, enabling long term storage, but these secretions are very scarce in female smooth newts (Boisseau & Joly 1975; Verrell & Sever 1988) compared with other urodeles and may explain the reduced storage times observed in smooth newts.

The smaller clutches of females only inseminated at the beginning of the season were laid during a shorter oviposition period compared with females that were re-inseminated, suggesting that females respond to depleted sperm supplies by ceasing to oviposit. Females appear to respond initially to diminishing sperm supplies by slowing their rate of oviposition, as the number of eggs laid during the later part of a female's oviposition period is reduced in comparison with the number of eggs laid in the early part of her oviposition period, and if they are subsequently unable to remate they cease oviposition altogether. Female newts are thought to continue yolking up and maturing medium-sized oocytes throughout the oviposition period, as more eggs are laid during a season than are estimated to be laid by counts of mature oocytes at the beginning of the season (Verrell *et al.* 1986; Harris 1987; Baker 1992a), and plasma vittellogenin levels increase during the reproduction period in *T. carnifex* (Zerani *et al.* 1991). Harris proposed that this mechanism may enable female *Notophthalmus viridescens* to make within-season adjustments of egg number based on resource availability, as egg production is costly. For example, Fitzpatrick (1973) found that 32% of the annual energy flow of female *Desmognathus ochrophaeus* is used in vitellogenesis. My results suggest that, in the absence of reinsemination, female smooth newts may refrain from maturing and ovulating further medium-sized oocytes. This mechanism may be mediated via estradiol which acts on receptors in the liver to initiate vitellogenesis (Ho 1991). In *Taricha granulosa*, courtship has been shown to induce the

release of estradiol; the concentration of estradiol in the plasma reaches a peak at sperm transfer and remains high for 24hr after the initiation of courtship (Propper & Moore 1991), but whether courtship induces the release of estradiol in smooth newts is unknown. Such a mechanism would not preclude females from reabsorbing mature eggs in the absence of sufficient sperm to fertilise the clutch, which occurs in other species in which sperm transfer is inefficient and unreliable, e.g. in the coral *Briareum asbestinum* (Brazeau & Lasker 1990). In either case, female smooth newts may be able to make seasonal adjustments to egg number in the absence of sufficient sperm to fertilise the entire clutch. Inadequate sperm numbers in the females' spermathecae may be a consequence of failure to acquire sperm under adverse environmental conditions or due to a paucity of males in small populations as females have been shown to prefer to mate with novel males (Hosie 1992).

The decrease in the viability of eggs (due to infertility and embryonic death) laid over time by females that are using sperm stored from the beginning of the season compared with the viability of eggs laid by females that gain fresh sperm, suggests that sperm quality deteriorates during storage in the spermathecae, which seems to be a common problem of sperm storage in females. Similar loss of fertility and increased embryonic death is correlated with the length of time that sperm are stored in the female reproductive tract in other taxa, e.g. in chickens (Lodge *et al.* 1971), in *Drosophila* (Pyle & Gromko 1978) and in grasshoppers *Eyprepocnemis plorans* (Lopez-Leon *et al.* 1994), suggesting that maintaining sperm viability is costly for females.

The mean oviposition period of female smooth newts inseminated only once was 18 days, compared with 45 days for females that were reinseminated. As female smooth newts begin ovipositing 6-10 days after initial insemination, these results suggest that by 23 days post-insemination the concentration of sperm remaining in the spermathecae may be below the threshold needed to maintain oviposition in female newts. This time interval is similar to the time taken for female smooth newts to become responsive to remating in an earlier study (approximately 20 days, Verrell 1984b), although a longitudinal study by Hosie (1992) found females mating at irregular intervals throughout the season. The mean oviposition

period (45 days) found in females reinseminated during this period corresponds reasonably well with the mean oviposition period seen in females with unlimited access to males (36.9 days, Baker 1992a).

The decline in egg-laying rate with time is similar in females inseminated only before the onset of ovulation and in females that were reinseminated during oviposition, suggesting that the spermathecae are probably filled to capacity after transfer of just one spermatophore and leakage or phagocytosis as well as sperm utilisation may occur. Females respond to diminishing sperm supplies by laying fewer eggs, and remating once during oviposition may still be insufficient for fertilisation of the full clutch, giving further weight to the hypothesis that females remate to replenish sperm supplies as a consequence of limited sperm storage capacity. Gabor & Halliday (1997) have shown that females remate with high-crested males in preference to gaining fresh sperm from low crested males. However, the interval between the initial insemination and remating was slightly shorter in their study and the number of eggs deposited by the females was not recorded, so the females may have still had sperm available for fertilisation. Taken together, their study and mine suggest that females use the need to replenish their sperm supplies to exercise mate choice after their initial insemination, but that when their supplies are very low they mate primarily to obtain sperm.

Over 40% of the matings involving either two males or one male and further courtship display did not result in oviposition, yet all the females in the study were inseminated during their first pairing with a male, suggesting that they were ready to mate and begin ovipositing. It seems unlikely that females would choose to totally forego reproduction for a season rather than lay eggs fertilised by an non-favoured partner as they may not survive until the next season. This finding suggests that female smooth newts may generally require more exposure to courtship display, or to exposure at closer time intervals than they received in this study to initiate oviposition. This conclusion is supported by Verrell's (1984b) study in which 17 out of 25 females, exposed to courtship display at varying intervals from 1 h to 48 h post insemination, laid at least a few fertile eggs from one spermatophore. In the

natural environment of the pond, a female choosing to remate probably has ample opportunity; certainly courtship in semi-natural ponds occurs on most days when the temperature rises above 8°C (Kauffmann pers comm, pers obs) throughout the season.

Transport of sperm into the cloaca and towards the apertures of the spermathecae in female *Notophthalmus viridescens* is mediated via contraction of the cloacal muscles (Hardy & Dent 1986a), which may allow females to choose whether to facilitate the transport of sperm into the spermathecae. In several species of bird (Birkhead & Møller 1992) and insects, e.g. in the fruit fly *Ceratitis capitata* (Yuval *et al.* 1996), differential filling of multiple storage tubules has been observed, suggesting that female choice for sperm storage may occur. Ward (1993) has suggested that female yellow dung flies *Scathophaga stercoraria* may be able to store sperm from different males in different spermathecal tubules and to release preferentially sperm from specific tubules to fertilise their eggs, but Simmons *et al.* (1996) tested this theory, also in the yellow dung fly, using experiments designed to control for male size and found no evidence for female control of paternity post-insemination.

The female tract may enable selection of 'vigorous sperm' (Birkhead *et al.* 1993) and multiple spermathecae may have evolved to enable females to preferentially utilise sperm from different males (Ward 1993), but whether the reproductive tract and multiple spermathecae of female smooth newts enables the preferential transport, storage and release of sperm for utilisation in fertilisation awaits investigation.

6.6 Summary

Onset of oviposition was not influenced by either the number of spermatophores picked up during a single encounter or the number of mates transferring sperm. 'Post insemination' exposure to courtship display from novel mates, in the absence of further insemination, significantly affected the proportion of females that started to oviposit. This finding supports the hypothesis that oviposition is induced by courtship display in female smooth newts.

Although, theoretically, one sperm mass contains sufficient sperm to fertilise a full clutch (Pecio 1992; chapter 4), the pattern of sperm utilisation in female smooth newts reveals that insemination by just one sperm mass fails to lead to fertilisation of the full clutch.

Females mated only before the onset of oviposition showed significantly reduced clutch size, duration of oviposition period and egg viability compared with females remated during the oviposition period. Therefore, temporal acquisition of sperm, through multiple mating, may be an important factor governing oviposition of the full clutch of eggs and enhancing viability of the offspring.

Females may mate multiply at the beginning of the season to induce ovulation, but this finding does not preclude multiply mating to guard against sterile sperm or to obtain 'better genes' through mate choice. These findings also suggest that females remate during the oviposition period to obtain fresh sperm supplies, probably because sperm storage is limited or because sperm lose their viability over time. Females may use the need to gain fresh sperm to exercise mate choice. These mechanisms need to be elucidated further. Assessment of offspring viability after remating females with either the same partner, or males in poor or good reproductive condition may differentiate between increasing viability by using younger sperm and increasing viability through mate choice. Similarly, quantitative estimates need to be made of the sperm storage capacity of the spermathecae, the amount of sperm present in the spermathecae after single and multiple insemination by one male, and the viability of sperm after different periods of storage.

Chapter 7. Physiological polyspermy in the ova of smooth newts

7.1. Introduction

I have shown previously (Chapter 4) that the number of sperm matrixed within one sperm mass of a male smooth newt is theoretically capable of fertilising the whole clutch of one female. However, this conclusion needs to be considered in the context of events occurring within the female reproductive tract. The female reproductive tract may be differentially selecting sperm in many species, and only a small proportion of the sperm transferred by a male during mating reach the site of fertilisation (Birkhead *et al.* 1993).

In smooth newts, two aspects of the female tract limit the number of sperm available for fertilisation; only live sperm enter the spermathecae and the spermathecae may not have the capacity to hold the full complement of sperm contained in one sperm mass (see Chapter 6 for further discussion).

Sperm are released at the time of oviposition of each individual egg; the spermathecal myoepithelium contracts, just prior to expulsion of the ovum from the oviduct, and expels sperm onto the ovum in the cloacal lumen (Fig 7.1). The orifices of the spermathecae are narrow and sperm may have to leave each spermatheca singly (Hardy & Dent 1986a). This theory is compatible with a study by Hardy & Dent (1987), which showed that female *Notophthalmus viridescens* possess 20-40 spermathecae and release 65±21 sperm in response to an ovum passing down the oviduct. The sperm were located in or near the openings of the spermathecae, so they may also be available to the next ovum to be fertilised

depending on the interval between the two depositions. Females often deposit an ovum, swim to the surface to breathe and return to the weed to continue oviposition (pers obs).

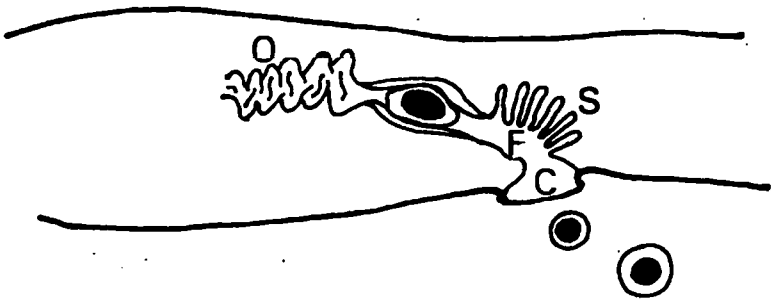


Fig 7.1 Fertilisation of ova in *Triturus vulgaris*. Just prior to deposition, an ovum leaves the oviduct (O) and passes the spermathecae (S), which release sperm onto the egg. The ovum is extruded from the cloaca (C) during deposition. Site of fertilisation =F (drawn in consultation with Sever).

Thus, of the many sperm that entered the spermathecae, only a small proportion may be available for each fertilisation, referred to as entering the 'fertilisation set'. Sperm release from the spermathecae, which is under neuromuscular control (Hardy & Dent 1987), may be triggered via pressure receptors responding to the presence of the ovum in the oviduct.

Verrell (1984c) reported a method of determining ovulation in female smooth newts by belly-pressing, a process that causes an ovum to be extruded from the cloaca of females carrying oviductal ova. Verrell demonstrated that these extruded ova were fertile, but subsequent workers have failed to observe any development (Baker pers comm; pers obs). Infertility of extruded ova may demonstrate that movement of an ovum down the oviduct and the subsequent sperm release is under female control, as females typically take five minutes to deposit a single ovum (Diaz-Paniagua 1982).

The sperm in the spermathecae may be exposed to phagocytosis, or ingestion by leukocytes during the oviposition period (see Chapter 6 for discussion), reducing the number of sperm available for fertilisation of the remaining ova. Phagocytosis may remove sperm that have degraded during storage, which may reduce the likelihood of embryonic mortality and reduce the physiological costs of storage.

Another factor which may also reduce the number of ova successfully fertilised is the occurrence of polyspermy. During sexual reproduction, two gametes, each containing a haploid complement of chromosomes, fuse to form a zygote. The zygote then carries the diploid chromosome set which is characteristic for that species. Genetic abnormalities can occur through polyspermy, when more than one sperm enters the ovum. Extra sperm may fuse with the egg nucleus (Fankhauser 1955) or may undergo DNA synthesis and form extra cleavage centres (Wakimoto 1979). Both of these scenarios result in aneuploidy (abnormal complements of chromosomes) and abnormal development. Polyspermic ova often die, but some survive to become offspring with obvious abnormalities. In the *Ambystoma jeffersonianum* complex the occurance of polyspermy and of fertile triploid hybrids leads to unusual chromosomal complements. The complex consists of two bisexual diploid species, *A. jeffersonianum* and *A. laterale*, and two female hybrid species, *A. platineum* which carries two complements of *A. jeffersonianum* and one complement of *A. laterale* chromosomes and *A. tremblayi* which carries one complement of *A. jeffersonianum* and two complements of *A. laterale* chromosomes (Duellman & Trueb 1994). The hybrid females produce triploid eggs in which the mechanism for rejecting extra sperm chromosomes does not function properly, so fertilisation results in either tetraploid individuals (*A. tremblayi* x *A. laterale*) in which all four chromosome complements replicate or diploid individuals in which both chromosome complements originate from the male (*A. platineum* x *A. jeffersonianum*) and the oocyte chromosomes fail to replicate. Cells and nuclei of the polyploid animals increase in size according to ploidy (Licht & Bogart 1987).

Many taxa, including anurans, exhibit monospermy, in which a single sperm enters the ovum and all subsequent sperm are excluded. The mechanism which prevents more than one sperm fusing with the nucleus of the ovum is termed the 'block to polyspermy' and occurs at two distinct levels, one termed 'fast' which occurs as the sperm penetrates the membrane and is transitory, and the other termed 'slow' which follows the 'fast' block and is more permanent. The 'slow' block involves the release of cortical granules into the perivitelline space, between the plasma and the vitelline membranes, that prevent sperm

binding to and penetrating through the vitelline membrane (Jaffe & Gould 1985; Wyrick *et al.* 1974; Elinson 1986; Cran & Esper 1990; Ducibella 1991).

In most urodeles, except Hynobiids (Iwao 1989), the penetration of the membrane by a sperm does not trigger the production of a membrane potential (Charbonneau *et al.* 1983); neither do cortical granules form (Elinson 1986). Hynobiids, therefore, are the only monospermic urodeles. In the rest of the urodeles, the block to polyspermy occurs in the cytoplasm in response to the fusing of the ovum pronucleus with a sperm pronucleus (Fankhauser 1934; McLaughlin & Humphries 1978; Elinson 1986; Iwao & Elinson 1990). Several sperm may pass through the membrane into the cytoplasm, but once a sperm pronucleus has fused with the pronucleus of the ovum, changes occur in the cytoplasm and the rest of the sperm pronuclei degenerate. This process is termed physiological polyspermy, to distinguish it from pathological polyspermy, which leads to abnormal development. Physiological polyspermy may be a consequence of females failing to evolve mechanisms that block polyspermy at the membrane level. Alternatively, polyspermy may directly benefit the developing zygote if the accessory sperm nuclei stimulate production of factors in the cytoplasm needed for development, a hypothesis that is supported in a study by Forfanova (1964, cited in Lorenz & Ogasawara 1968), which found that too few sperm at the site of fertilisation interferes with development.

7.1.1. Polyspermy in urodeles

In urodeles, the mechanisms of physiological polyspermy have been investigated quite widely. All the sperm entering the cytoplasm of newt ova form pronuclei and undergo DNA synthesis (Wakimoto 1979; Iwao *et al.* 1985). Each sperm nucleus travels through the cytoplasm, at first independently, but after 15 minutes the nucleus is surrounded by a fast-growing sperm aster of microtubules that prevents each sperm from interacting with another sperm (Fankhauser & Moore 1941). The sperm aster produces a central area of yolk-free cytoplasm within which the sperm nucleus decondenses, and the first sperm aster to contact the ovum nucleus fuses with it. The ovum nucleus controls activation of maturation-promoting factor, MPF. The concentration of MPF increases around the fused pronuclei,

causing the concentration of MPF to decrease in the rest of the cytoplasm. The reduced levels of MPF lead to degeneration of the accessory sperm pronuclei (Iwao *et al.* 1993) before the first cleavage of the zygote occurs.

In the ctenophore *Beroe ovata*, the situation is fascinating. Several sperm enter the ovum and the ovum nucleus travels around in the cytoplasm and inspects each sperm nucleus before fusing with one (Carré & Sardet 1984), which may be an example of cryptic female choice. Whether this occurs in urodeles is unknown.

Sperm entry points on the plasma membrane of newt ova, including the ova of *Triturus vulgaris* (Semik & Kilarski 1986), can be seen as areas of high densities of microvilli under scanning electron microscopy (SEM). Using this technique to follow the appearance of sperm entry sites, fertilisation has been shown to occur between three and ten minutes after the ovum has passed the orifices of the spermathecae in *Notophthalmus viridescens* (Fankhauser & Moore 1941; McLaughlin & Humphries 1978) and in *Pleurodeles waltl* (Picheral 1977). Consequently, the number of sperm entering the ovum can be assessed after the ovum has been deposited by the female. The jelly hydrates on contact with water, which limits the time available for fertilisation; ova of both *N. viridescens* (McLaughlin & Humphries 1978) and *Cynops pyrrhogaster* (Matsuda & Onitake 1984) cease to be fertilisable after 15 minutes in water. In Hynobiids that exhibit external fertilisation, the ova remain fertilisable for up to three hours after contact with water (Hasumi *et al.* 1993).

7.1.2. Levels of polyspermy

In urodeles, levels of polyspermy have been investigated in three families of salamanders, the Ambystomatidae, the Plethodontidae and the Salamandridae. In most species examined, one to 30 sperm may enter a single ovum, although the modal range is two to eight (Table 7.1). High numbers of sperm per ovum, e.g. more than ten sperm in *Triton palmatus* (now classified as *Triturus helveticus*, Fankhauser 1932), more than 13 sperm in *Notophthalmus viridescens* (Kaylor 1937) and more than 30 sperm in *Cynops pyrrhogaster* (Iwao *et al.* 1985), usually results in irregular cleavage and death. Similarly, high numbers of sperm at

the site of fertilisation leads to embryonic mortality in chickens, which also exhibit physiological polyspermy (Ogasawara & Lorenz 1966). Introducing lower numbers of sperm to the site of fertilisation reduces the level of embryonic mortality, showing that mortality is due to polyspermy and not due to abnormal sperm, which would normally fail to traverse the female tract, being present in the upper oviductal tract (Lorenz *et al.* 1969; Lake 1975; Birkhead & Møller 1992).

Table 7.1 Levels of polyspermy in urodeles.

species	**No of sperm range (modal range)**	**method**	**reference**
Notophthalmus viridescens	1-19 (2-6)	sectioning ova, natural mating	Fankhauser & Moore 1941
	1-13 (4-8)	penetration marks	Kaylor 1937
	1-30 in jelly (6) in cytoplasm	sectioning ova, natural mating	McLaughlin & Humphries 1978
Cynops pyrrhogaster	1-32 (4)	penetration marks	Streett 1940
	2-20	not stated	Iwao *et al.* 1985
Triturus helveticus	1-9 (2-4)	penetration marks	Fankhauser 1932
Pleurodeles waltl	3-15	penetration marks, natural mating	Charbonneau *et al.* 1983
Ambystoma mexicanum	10-20	artificial fertilisation	Charbonneau *et al.* 1983
Hynobius nebulosus	1 (monospermic)	pigment granule, fertilisation potential	Iwao 1989

In *Cynops pyrrhogaster*, the sperm nuclei in some highly polyspermic ova fail to decondense and form asters, resulting in fertilised eggs which do not develop at all and thus appear to be unfertilised when checked by eye (Iwao *et al.* 1985). Normal development has been observed in ova containing 16 and 32 accessory sperm pronuclei in *Triturus alpestris* (Fankhauser & Moore 1941) and in *C. pyrrhogaster* (then classified as *Triturus pyrrhogaster*, Streett 1940) respectively. These findings led Fankhauser and Moore (1941) to propose that species with larger eggs are able to tolerate higher levels of polyspermy before abnormal development occurs.

7.1.3. Structure of urodele sperm

The structure of urodele sperm is well described, e.g. in *Pleurodeles waltl* (Picheral 1979), in *Notophthalmus viridescens* (Fawcett 1970), in 11 species of terrestrial salamanders (Wortham *et al.* 1982) and in 15 species of Japanese salamanders (Kuramoto 1995). Urodele sperm are very long, ranging from $250x10^{-3}$ mm in *Pleurodeles waltl* to 1.0 mm in *Necturus maculosus*, and can be easily observed at x200 magnification. The sperm of urodeles differ from those of other vertebrates in that the tail possesses a flagellum attached via an undulating membrane. Sperm from different species of urodeles are very similar and consist of the following structures: the acrosome, head, neck piece and tail (Fig 7.2). The acrosome at the very tip of the sperm possesses a sharp tip and a recurving barb. It is not known what the precise function of the barb is, but the acrosome is involved in penetration of the ovum and contains lytic enzymes to dissolve the membrane (Picheral 1979). The head contains the nucleus of condensed chromatin and is connected to the tail via the neck piece, which consists of a long cylinder with a characteristic 'step down' in diameter between the head and the tail. The tail, which is between two or three times longer than the head, comprises two distinct areas, the mid-piece and the principal piece. The undulating membrane traverses the full length of the tail and extends some way beyond. The mid-piece of the tail is surrounded, except at the point of attachment of the undulating membrane, by a mitochondria-rich sheath.

7.1.4. Structure of the ovum of urodeles

The ovum of urodeles consists of an animal hemisphere, which is darkly pigmented, and a vegetal hemisphere, which is pale in contrast. The ovum, surrounded by a plasma and a vitelline membrane, is released from the follicle. As the ovum passes down the oviduct, each region secretes a gel with differing composition and morphology so that the ovum entering the cloaca is surrounded by five layers of mucopolysaccharides and glycoproteins which form the jelly, described in *Notophthalmus viridescens* (McLaughlin & Humphries 1978).

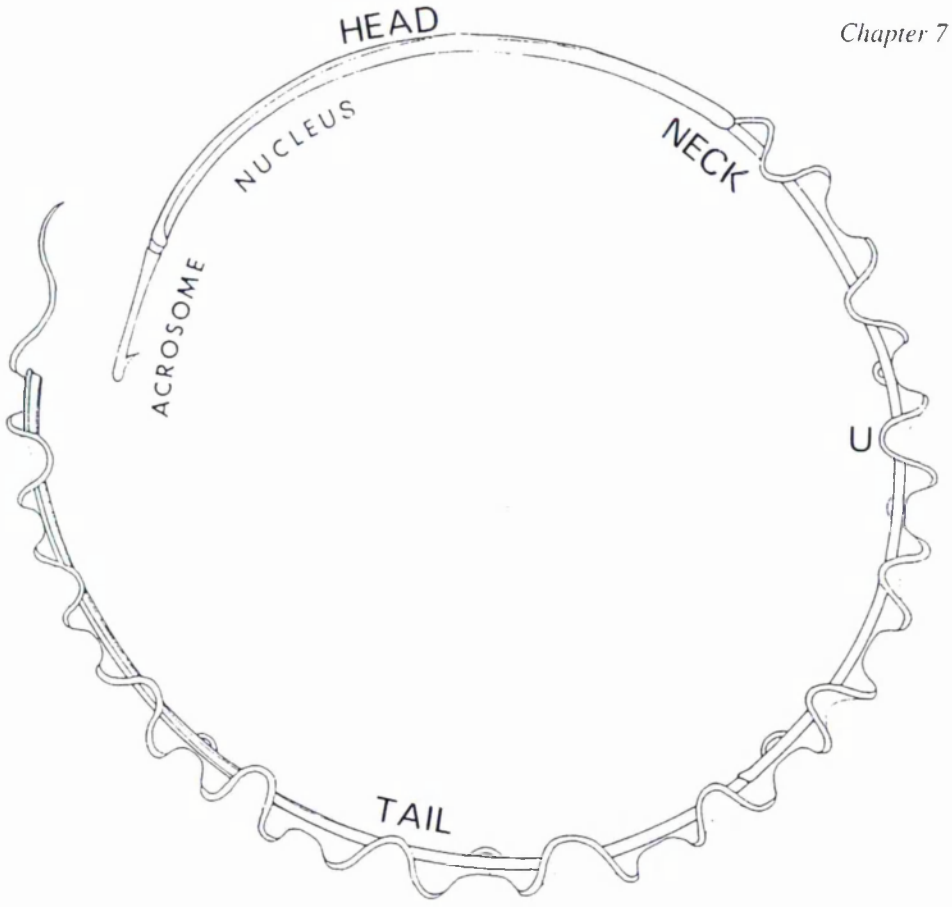


Fig 7.2 Drawing of urodele sperm (after Picharel 1979). The tail possesses an undulating membrane (u).

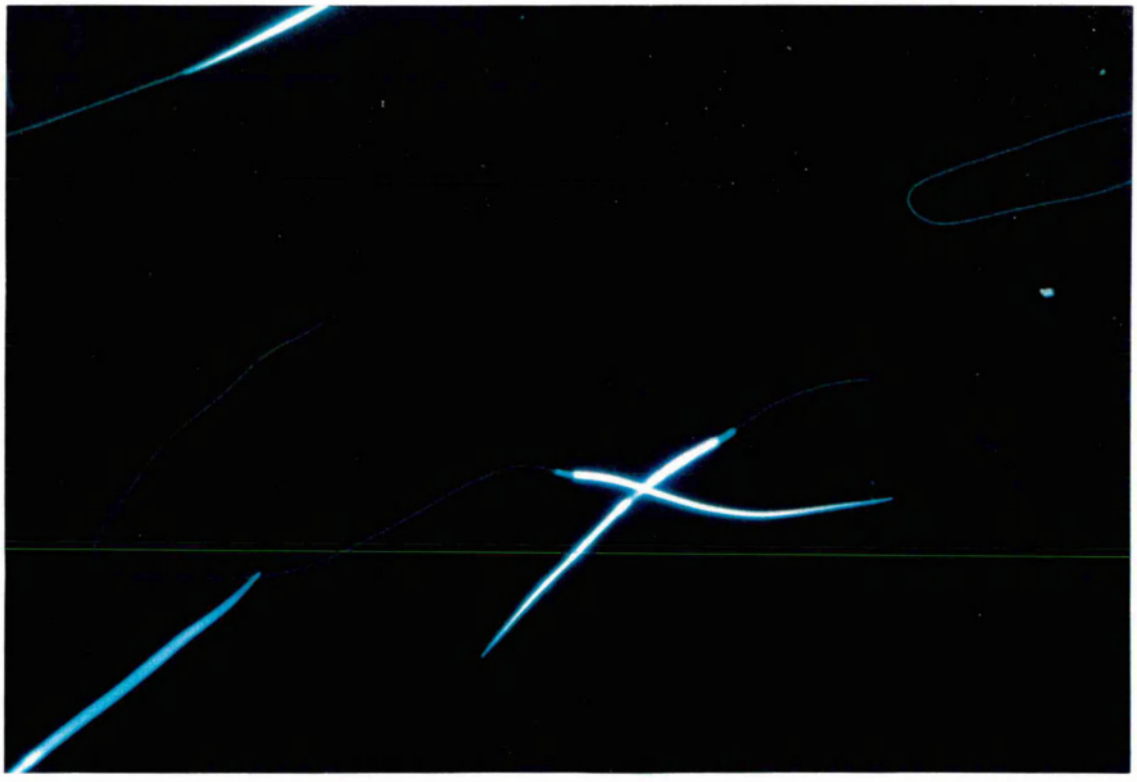


Fig 7.3 Sperm of *Triturus vulgaris*, stained with bis-benzimide and viewed under fluorescent microscopy. (x400Magnification)

Sperm do not fertilise jellyless eggs (McLaughlin & Humphries 1978), although Matsuda and Onitake (1984) have succeeded in fertilising jellyless *Cynops pyrrhogaster* ova using sperm in a high salt solution, leading these authors to propose that the jelly layers may provide the correct ionic environment for fertilisation. Recent studies in other species have shown that mucopolysaccharides aid in cell recognition and that specific receptors on sperm bind to sugar moieties on the ovum surface prior to fertilisation (Dow *et al.* 1996). Thus the jelly layers may also direct the sperm towards the ovum, may assist in the recognition of conspecific sperm and may activate the sperm ready for fertilisation. The ovum nucleus is present in the animal hemisphere (Fankhauser 1948; Slack 1983), but sperm enter both the animal and vegetal hemispheres of urodele ova (Fankhauser & Moore 1941), whereas in anurans the sperm only enter the animal hemisphere (Elinson 1986).

7.2. Aims

This study aims to investigate the level of physiological polyspermy which occurs in naturally deposited ova of smooth newts and to determine whether the time taken to deposit an ovum is related to successful fertilisation. The influence on fertilisation of the time each ovum is retained in the cloaca will be investigated by comparing the proportion of ova fertilised during natural oviposition with the proportion of ova fertilised during belly pressing.

Hypothesis: H_1 Females spend a long time depositing each ovum (approximately five minutes) to allow sperm access to the cytoplasm of the ovum.

prediction : 1 Naturally deposited ova will be more likely to be fertilised than ova extruded from the cloaca of females by belly-pressing.

Hypothesis: H_2 Time spent in the cloaca prior to oviposition is an important factor in the level of polyspermy.

prediction : 2 Naturally deposited ova will contain more sperm than ova expressed by belly-pressing.

7.3. Methods

7.3.1. Collection of naturally deposited ova

Ova need to be collected within five minutes of sperm release to ensure that the DNA in the sperm heads has not begun to decondense and change the shape of the sperm, making identification difficult. Therefore, each ovum must be collected immediately after deposition.

Female newts were captured from the wild and housed in individual tanks containing strips of artificial weed (as described in Chapter 2) in an unheated garage. Prior to each observation period, fresh weed was placed into the tanks so that each recently laid ovum could be readily identified. On many evenings during the breeding seasons in 1994 and 1995, the females were watched from dusk to 11 pm and each ovum was collected as the female released the weed after each deposition. It is essential to wait until the female swims away from the weed otherwise she may be disturbed prior to actually depositing an ovum. A female may spend 15 minutes in oviposition, motionless, holding the weed to her cloaca with her back legs, before releasing the weed. Only one ovum is deposited at a time and, on releasing the weed, the female goes up to the surface to breathe. Occasionally, a female failed to deposit an egg, and on two occasions the jelly mass was present but did not contain an ovum. No jellyless eggs were ever found. The strip of weed was removed from the tank and the fringe containing the egg was cut off and placed into 0.4% formaldehyde. The ova were stored prior to determination of the number of sperm present. The strip was then replaced, although, usually, the collection of the egg had disturbed the female and she did not begin ovipositing again for some time. One egg was collected from each of 30 females.

7.3.2. Collection of ova extruded by belly-pressing

Females were netted from a pond once a week as part of a different study and tested for onset of ovulation by belly-pressing. Ova were collected from 23 females and each expressed ovum was placed in 0.4% formaldehyde and stored as above.

7.3.3. Determination of number of sperm present in an ovum

As discussed in the introduction, many researchers determine the number of sperm entering the ovum by counting the sperm entry sites, identifiable as areas with high densities of microvilli under scanning electron microscopy (SEM). This method works very well for anuran ova in which the sperm only enter the animal pole, but I found it an unsatisfactory method for urodele ova in which sperm enter all over the surface of the ovum. Counting the number of entry sites in urodele ova requires the ovum to be viewed from both sides under SEM. Unfortunately, when trying to reposition the ovum to view the second surface, the ova repeatedly crumbled. Finally, I decided to use a different approach.

I modified a technique which has been developed to observe bacteriophage DNA injected into *Xenopus* eggs (Forbes *et al.* 1983). The DNA is stained with bis-benzimide (Hoescht 33258) and can be observed under fluorescence microscopy. The fluorochrome solution consists of $10x10^{-6}$ g ml^{-1} bis-benzimide in buffer (15 mM Pipes, 2 mM EDTA, 2 mM EGTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermidine, 0.2 mM spermine, 0.5 M sucrose, pH 7.2). To ensure that individual sperm in an ovum could be identified, the technique was evaluated using sperm in sperm masses. The sperm mass was placed onto a glass slide and $10x10^{-3}$ ml of fluorochrome solution was pipetted over it. A cover slip was placed on top to prevent the sperm mass drying out. The slide was viewed under phase contrast, and most of the sperm could be observed tightly coiled together within a matrix. However, some sperm were freed by the squash, and the complete sperm, both head and tail, could be clearly identified. The slide was then viewed under fluorescence and both the head and tail of the sperm were observed to fluoresce, probably due to the DNA in the mitochondria-rich sheath around the mid-piece of the tail (Fig 7.3). Newt sperm can be easily recognised under fluorescence, so I used this method to scan the cytoplasm and the jelly layers of the ovum for sperm.

The ovum was placed on a glass slide and the jelly layers removed, using two hypodermic syringe needles, and moved to one edge of the slide. $10x10^{-3}$ ml of fluorochrome solution

was pipetted over the ovum and the jelly layers and the ovum was lysed by the addition of a coverslip (22x50 mm). Addition of the coverslip also flattened out the jelly layers. The number of sperm that had entered the ovum and the jelly layers was determined by 'scanning' the slide under fluorescence. The slide was calibrated using a stage graticule and each 1 mm travel of the stage was equal to a complete field of view at x200 magnification. Therefore, any sperm present in one scan were counted and none were missed by the next scan travelling too far down the slide. The slide was scanned horizontally across the full length of the coverslip, and the identity of each sperm was confirmed by viewing at x400 magnification.

7.4. Results

7.4.1. Individual sperm in the ovum

Some sperm in the jelly were straight, while other sperm were curved or even coiled. Picheral (1979) has shown that sperm in the outer and middle viscous jelly layers of the ovum are straight, whereas those in the inner jelly layers and the capsular chamber take up a curved configuration. He proposes that this is due to ionic differences in the different layers. Many of the sperm in the cytoplasm of the ova consisted of only the head and neck, the break appearing behind the 'step-down' between the neck and the tail.

7.4.2. Number of sperm entering an ovum during natural deposition

The number of sperm in an ovum was determined for one ovum deposited by each of 30 different females. The majority of the ova contained low levels of sperm, range 1-9, although some ova contained up to 20 sperm, one contained 54 and another more than 100 sperm (Fig 7.4). Four ova didn't contain any sperm in either the ovum cytoplasm or the jelly layers. When the four ova that did not contain any sperm and the two ova containing high levels of sperm were excluded from the analysis, the mean number (± the standard deviation) of sperm entering the ovum cytoplasm was 3.10±1.95 and entering the jelly layers was 2.8±1.60 (n=24). Assuming that the sperm in the jelly layers could have still entered the ovum if the ovum hadn't been fixed gives a mean value of 4.23±2.37 sperm

per ovum. Although many of the sperm were distributed singly throughout the cytoplasm of the ovum, in some cases sperm were found clustered together, especially in ova containing higher numbers of sperm. Clusters of 2-5 were common in the ova containing 8 or more sperm, and the ova containing 54 sperm and more than 100 sperm contained sperm clusters of 5-11 sperm. Pieces of a transparent matrix containing sperm, which were similar to the matrix of a sperm mass, were observed on the jelly layers of the ovum containing more than 100 sperm.

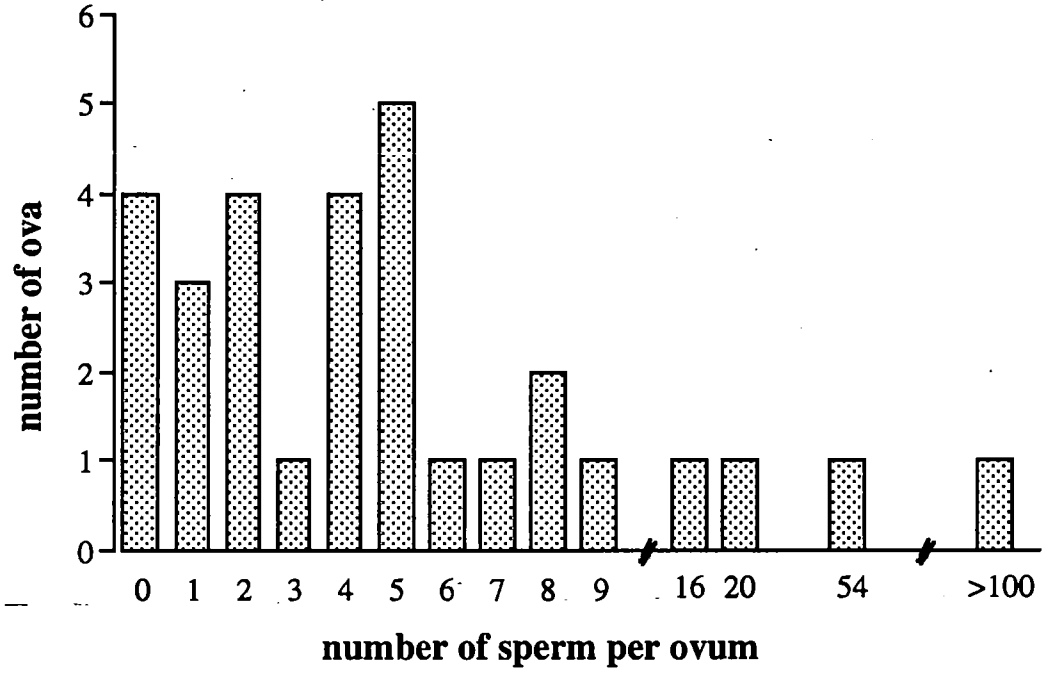


Fig 7.4 Number of sperm entering individual ova during natural deposition.

Most of the sperm entering the cytoplasm of the ova had lost their tail section, although there was no difference between the number of intact sperm and the number of sperm heads in the jelly layers (Fig 7.5).

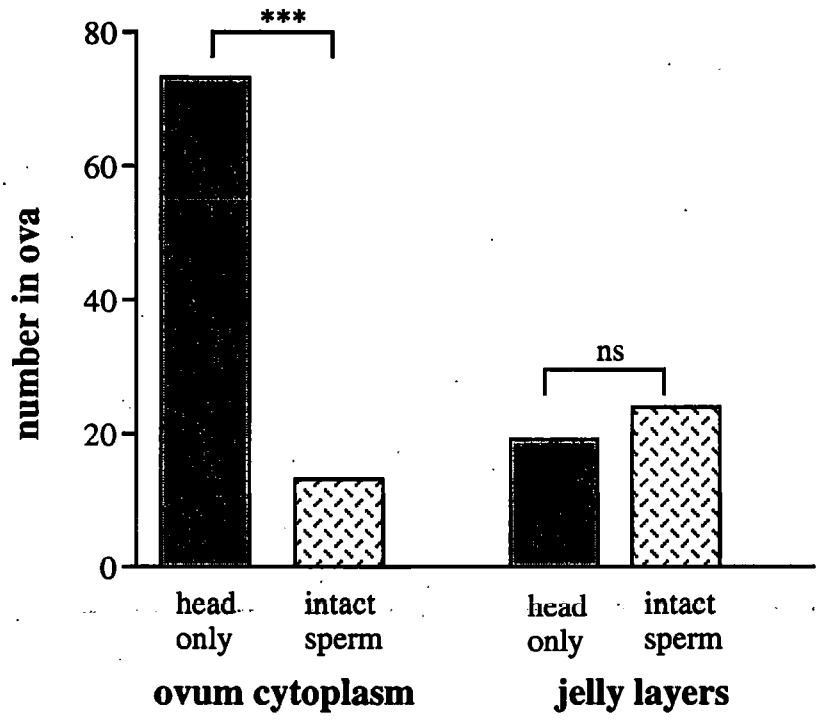


Fig 7.5 Comparison of the number of intact sperm and sperm heads found in the ovum matrix and the jelly layers of naturally deposited ova containing between 1-20 sperm (Wilcoxon matched pairs: n=24, ovum cytoplasm: z=-3.70, p=0.002***; jelly layers: z=-0.70, p=0.48 ns=non significant).

7.4.3. Number of sperm entering an ovum during belly-pressing

Nine of the 23 ova contained no sperm in either the jelly layers or the cytoplasm and three ova contained more than 30 sperm (Fig 7.6). Clusters of sperm were also observed in extruded ova; ova containing between 6-30 sperm had clusters of 6-12 sperm. Both the ova with more than 100 sperm contained many clusters including one containing 18 sperm. The ovum with more than 135 sperm contained two coiled clusters of sperm on the jelly layers, in which individual sperm could not be clearly identified and counted. When the 12 ova containing either no sperm or very high numbers of sperm were excluded from the analysis, the mean number (±s.d.) of sperm in the ovum cytoplasm was 1.29±0.76 and in the jelly layers was 5.10±5.09, giving a mean value of 3.54±4.30 sperm per ovum (n=11).

The number of sperm entering an ovum during belly-pressing, when only ova containing average levels of sperm (1-20) were considered, did not differ significantly from the number of sperm entering an ovum naturally (Mann-Whitney U-test: n_1=11; n_2=24; z=-0.93, p=0.35). However, when all the ova were considered, significantly more ova were

fertilised during natural oviposition than were fertilised during extrusion from the cloaca of females by belly pressing (Fig 7.7).

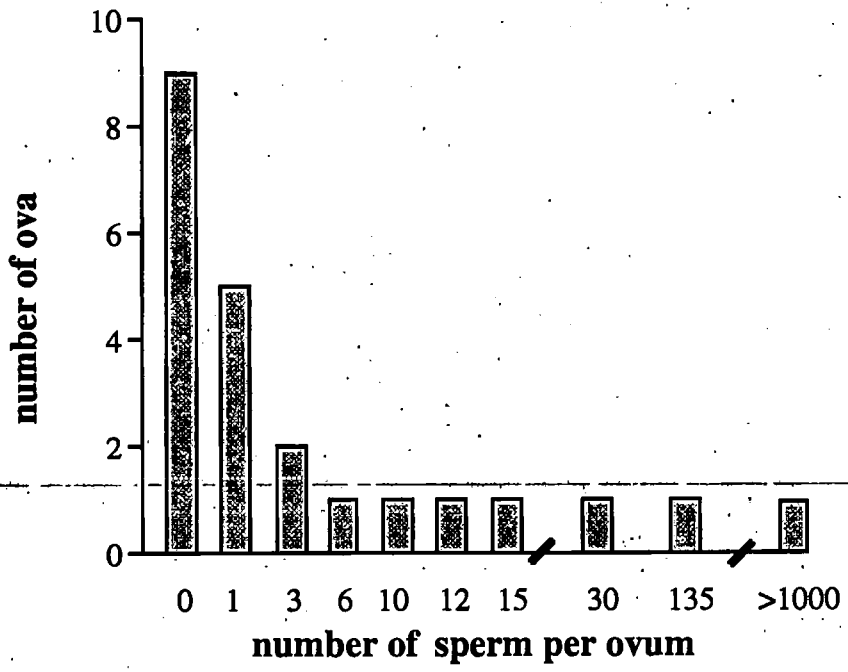


Fig 7.6 Number of sperm in ova extruded by females in response to belly-pressing.

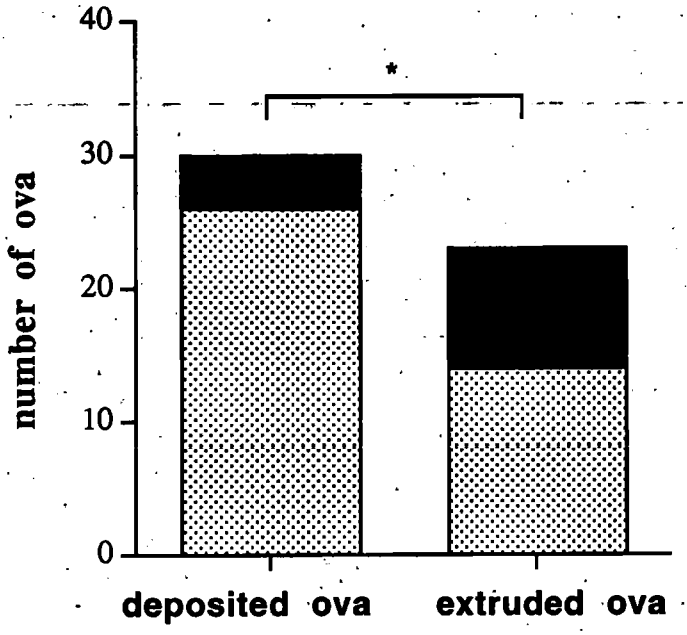


Fig 7.7 Comparison of the number of fertilised [dots] and unfertilised ova [black] that are deposited, or extruded by belly-pressing (G-test: n_1=30, n_2=23, df=1, g=4.70; $p<0.05^*$).

7.4.4. Distribution of sperm between ovum cytoplasm and jelly layers in deposited and extruded ova

Deposited ova contained significantly more sperm in the ovum matrix than in the jelly layers, whereas there was no significant difference in the distribution of sperm in extruded ova (Wilcoxon paired sample test: $n_{oviposited}$=24, z=-2.014, p=0.044; $n_{extruded}$=12, z=-0.94, p=0.35). However, ova with high numbers (>30) of sperm contained very few sperm in the cytoplasm (Table 7.2).

Table 7.2 Distribution of sperm between cytoplasm and jelly layers in ova containing high numbers of sperm

total number of sperm in ovum	number of sperm in cytoplasm	number of sperm in jelly layers
a) in extruded ova		
30	1	29
135	1	134
>1000	10	>1000
b) in deposited ova		
54	3	51

7.5. Discussion

7.5.1. Levels of polyspermy

The number of sperm entering individual ova in *T. vulgaris* is comparable to the number of sperm entering the ova of other urodeles (Table 7.1). The proportion of sperm consisting of heads only is higher in the cytoplasm than in the jelly layers, which suggests that the tail is lost as the sperm head passes through the ovum membrane. Although the loss of the tail may be an artefact of the methodology, other researchers have previously concluded that the tail of urodele sperm may not enter the ova. Fankhauser and Moore (1941) reported that when ova were sectioned serially, tail-like filaments were only seen in or near the paths of penetration in two cases. Similarly, McLaughlin and Humphries (1978) could not clearly distinguish the tail in living specimens observed microscopically, although the head and neck piece were easily identified.

In my study, five of the ova examined contained large numbers of sperm. Artificial insemination trials have demonstrated that the number of sperm entering an ovum is proportional to the concentration of sperm surrounding the ovum, suggesting that urodeles have no mechanism to prevent sperm entry. In trials using freshly removed ova and high concentrations of sperm, more than 30 sperm entered ova in *Cynops pyrrhogaster* (Iwao *et al.* 1985) and up to 60 sperm entered ova in *Pleurodeles waltl* and in *Ambystoma mexicanum* (Charbonneau *et al.* 1983). Extremely high levels of polyspermy can be induced; the maximum number of sperm recorded entering an ovum of *C. pyrrhogaster* by artificial insemination is 800. The studies by Charbonneau *et al.* (1983) and Iwao *et al.* (1985) suggest that the ova in my study which contained large numbers of sperm may have been exposed to higher densities of sperm in the cloaca of the female. Higher densities of sperm surrounding an ovum may be a consequence of differential release of sperm from the spermathecae, as females may use sperm with low efficiency soon after mating and with higher efficiency as time after mating increases (Birkhead *et al.* 1993). This hypothesis may be supported in urodeles as the level of polyspermy in *Cynops pyrrhogaster* ova (Streett 1940) and the number of sperm released in response to neurotransmitters and hormones in *Notophthalmus viridescens* (Hardy & Dent 1987) differs between individual females, which may correspond to females with fuller spermathecae releasing more sperm per ovum. Alternatively, individuals may store different amounts of sperm, e.g. if the size of the spermathecae varies between individuals.

Recently inseminated females have high levels of sperm in the cloaca for about one hour after mating (Hardy & Dent 1986a). Therefore, the ova containing more than 100 sperm may have been laid by recently inseminated females. This conclusion is further supported as traces of a matrix containing sperm were found on the jelly layers of these ova, which appeared to be similar to the matrix observed in freshly deposited sperm masses. In addition, the ovum containing more than 1 000 sperm had two coils of sperm on the jelly layers.

Despite highly polyspermic ova tending to cease development, ova which are penetrated by very high numbers of sperm may still hatch normally. McLaughlin and Humphries (1978) have shown in *Notophthalmus viridescens* that once the ovum begins hydration, jelly layer three becomes impermeable to sperm. Therefore, most of these excess sperm may be prevented from entering the ovum cytoplasm.

7.5.2. Are naturally deposited ova more likely to be fertilised than ova extruded from the cloaca of females by belly-pressing *(prediction 1)* ?

This study demonstrated that a higher proportion of naturally deposited ova are fertilised compared with ova extruded from females during belly-pressing, suggesting that sperm release is under female muscular control and is not just a consequence of the ovum passing the openings of the spermathecae. Other studies have also shown that infertile ova are naturally deposited by females during the season (Pecio 1992; Chapter 6). Infertility was determined by lack of development, and it therefore remains to be established whether these ova are truly unfertilised, i.e. contain no sperm, or are actually highly polyspermic ova which have failed to develop (Iwao *et al.* 1985).

7.5.3. Do naturally deposited ova contain more sperm than ova extruded from the cloaca of females by belly-pressing *(prediction 2)* ?

Although fewer ova were fertilised during belly-pressing than during natural deposition, the number of sperm in ova fertilised during belly-pressing did not differ significantly from the number of sperm in ova fertilised naturally. It appears, therefore, that the ova extruded from the cloacae of females by belly-pressing may fall into two classes, namely; ova that were ready to be deposited and, consequently, had evoked the release of sperm, and ova that were higher up the oviduct and had not yet evoked the release of sperm. The ova higher up in the oviduct at the time of capture may be extruded unfertilised or may be covered in sperm as they pass through the cloaca during belly pressing. Not only would such a possibility explain the lack of difference between the sperm numbers present in the ova that were actually fertilised during the two scenarios, but it would also explain the observed

distribution of sperm. Ova fertilised during natural deposition contained significantly more sperm in the cytoplasm than in the jelly layers, whereas the sperm were more evenly distributed between the cytoplasm and the jelly in ova fertilised during belly pressing or were concentrated in the jelly layers.

My findings suggest that some sperm reach the ovum cytoplasm quickly and the rest penetrate more slowly. Similarly, in *Notophthalmus viridescens*, some sperm may reach the ovum cytoplasm within one minute, although most sperm are entering jelly layer four at this time (McLaughlin & Humphries 1978). Whether these fast penetrating sperm are more likely to fertilise the ovum awaits investigation. Female smooth newts may regulate the number of sperm coming in contact with an ovum, using sperm more efficiently as sperm supplies become depleted, and may exercise cryptic female choice if sperm from different males can be preferentially stored and released (Lessells & Birkhead 1990). Cryptic female choice in smooth newts is an exciting area for future research (see also Chapter 6).

These findings support the hypothesis that the time taken by the female to deposit an ovum is to ensure that fertilisation occurs before the ovum leaves the female's cloaca.

7.5.4. The effect of polyspermy on male and female reproductive success

Sperm masses contain between 38 000 and 148 000 sperm (Chapter 4). Therefore, a single insemination should be sufficient to ensure fertilisation of a full clutch of 600 eggs, even when polyspermy levels of 1-20 sperm per ovum are taken into account. However, estimates of this kind fail to take into account other factors which may affect the efficiency of fertilisation. Sperm storage is known to be limited in some newt species, and the occasional very high level of polyspermy (100-1 000) found in this study suggests that ova may be penetrated by high levels of sperm if the concentration of sperm in the cloaca is higher than usual, e.g. when a female has mated recently. Thus if a female continues to oviposit, after insemination, many sperm may be lost before they can reach the spermathecae. Combating sperm loss may be an explanation for why multiple insemination of a single female occurs in smooth newts. Transfer of high levels of sperm may ensure that sufficient sperm remain in

the cloaca to saturate the spermathecae, even if insemination is quickly followed by deposition of an ovum.

Polyspermy, possibly in conjunction with phagocytosis of sperm and loss of sperm viability during storage, may contribute to females depleting their sperm supplies before the full clutch has been laid (chapter 6). Thus more accurate estimates of the number of sperm that can be stored in the spermathecae need to be obtained before the impact of polyspermy on fertilisation of a clutch can be determined. Consequently, although this study aimed to assess the possible influence of polyspermy on male reproductive success after transfer of one spermatophore, such an approach was too simplistic in the light of results from the study of sperm utilisation in females (Chapter 6).

7.6. Summary

In keeping with other urodeles, smooth newts exhibit physiological polyspermy, in which more than one sperm enter an ovum but only the nucleus of one sperm fuses with the ovum nucleus. Typically, 1-20 sperm enter each ovum, although the median range is 1-9. In naturally deposited ova exhibiting this level of polyspermy more sperm are found in the cytoplasm than in the jelly layers.

Much higher levels of polyspermy can occur, although the majority of the sperm are then found in the jelly layers rather than in the cytoplasm, which may be a consequence of additional sperm being trapped in the jelly layers as the jelly layers change in response to hydration. The occasional very high level of polyspermy (100->1 000) suggests that ova may be penetrated by high levels of sperm if the concentration of sperm in the cloaca is higher than usual, e.g. when a female has mated recently and the majority of the sperm are still in the cloaca.

The time each ovum spends in the cloaca, prior to oviposition, may be important in ensuring that each ovum is fertilised, and may explain why females spend up to 5 minutes depositing each ovum.

Chapter 8. Final Discussion

This study has focused on two aspects of the reproductive biology of smooth newts, spermatophore production in males and sperm utilisation in females, which, in addition to mate acquisition and gamete production, may influence the reproductive success of individuals of both sexes. This chapter discusses the findings in relation to the mating patterns exhibited by *T. vulgaris* and the reproductive strategies exhibited by other urodeles.

Many studies have investigated social factors, such as mate availability (Davies 1991) and ecological factors, such as resource distribution and temperature (Emlen & Oring 1977; Clutton-Brock 1988; Verrell 1989; Sullivan, Ryan & Verrell 1995) that may influence the mating pattern of a population, yet physiological factors that constrain reproduction, either directly or as a consequence of life history factors that differentially limit the physiology of individuals, may exert a greater influence (Halliday 1987; Vehrencamp & Bradbury 1984). For example, environmental conditions may be conducive for mating and potential mates may be readily available, yet an individual male newt is constrained if he is unable to produce a spermatophore or to display to a female. Similarly, a female newt is constrained if she has run out of viable sperm or her ova are still immature when the environmental conditions are favourable for oviposition.

Physiological factors, such as the need to replenish resources after mating, may remove an individual from the population of available mates for a period of time (Johnstone *et al.* 1996). The proportion of time that an individual is available or unavailable to mate is

termed his or her potential reproductive rate (PRR). The sex with the lower PRR limits the mating opportunities of the sex with the higher PRR, skewing the operational sex ratio (the ratio of sexually active males to fertilisable females, Emlen & Oring 1977) which may lead to competition among individuals of the faster sex (Clutton-Brock & Vincent 1991; Clutton-Brock & Parker 1992; Kvarnemo & Ahnesjo 1996). Physiological factors in amphibians which may affect the PRR of a given sex include their oxygen capacity, e.g. in *T. vulgaris* (Halliday & Sweatman 1976) and the energetic costs of calling, e.g. in tree frogs *Hyla versicolor* (Wells & Taigen 1986).

8.1. Mating patterns in smooth newts

In the wild, male competitive interactions increase and courtship encounters correspondingly decrease after the synchronous onset of ovulation (Verrell & McCabe 1988), suggesting that the operational sex ratio becomes male-biased during oviposition. These authors propose that the change in bias may reflect a decrease in the females' motivation to mate (Verrell & McCabe 1988). In this discussion I shall argue that the level of motivation to mate, expressed by individuals of either sex, may be influenced by physiological factors that limit their potential reproductive rate.

Although both male and female newts mate multiply during the breeding season, the reproductive strategies differ between the sexes. In the laboratory, male newts deposit spermatophores during standardised encounters on many days throughout the season (Halliday 1976; chapter 5), even though variation exists among males in the timing of mating and the quantity or quality of their spermatophores, which may be related to their body condition, reproductive condition, body size and the quantity and quality of the food available (chapter 5). However, female newts in the laboratory mate infrequently, between two and five times during the season (Hosie 1992). The majority of their matings occur before the onset of oviposition. At this time, females actively solicit sperm by initiating courtship encounters (Hosie 1992; Waights 1996) and by interfering in on-going courtships (Waights 1996). My findings suggest that, prior to the onset of oviposition, females may mate multiply to induce ovulation through exposure to male courtship display, but this

does not preclude the possibility that females also mate multiply to avoid laying infertile ova through mating with infertile males, to provoke sperm competition between the sperm of different males and thus acquire the fittest sperm to fertilise their offspring, or that they mate sequentially with fitter males to acquire the fittest sperm for fertilisation (discussed in Chapter 6).

Before the onset of ovulation, male sexual interference occurs on relatively few occasions (Verrell & McCabe 1988; Waights 1996), which may be a consequence of physiological constraints limiting male mating capacity when many receptive females are available. Male mating capacity may be limited by one or more of the following factors:-
a) the maximum number of spermatophores that a male is able to deposit during a single evening (Halliday 1976; Verrell 1986a; Baker 1990a; chapter 5);
b) the quality of second and subsequent matings, as both sperm number per sperm mass (chapter 4) and the number of sperm masses picked up by a female may be reduced (chapter 5);
c) the requirement for a behavioural recovery period after mating to sexual exhaustion (Verrell 1984a), which may be regulated by the time taken to replenish the secretory products in the cloacal glands (chapter 3).

Males may sire a higher number of offspring if they inseminate several females during an evening, even though the number of sperm transferred during later matings may be reduced (chapter 4). Similarly, unmated or once-mated females may be motivated sufficiently to mate with depleted males. Therefore, prior to oviposition, the potential reproductive rate of males may be lower than that of females due to the constraints of spermatophore production, linked to female's high requirement for sperm. Consequently, female competition for mates may occur, which is known to occur in the wild in *Triturus boscai* (Faria 1995) and is also attributed to females showing high motivation to mate.

After the onset of oviposition, remating in female newts may be determined primarily by intrinsic physiological factors rather than by extrinsic environmental factors. In a semi-

natural population, the degree of mating activity exhibited by female newts, unlike male newts, was not correlated with water temperature (Kauffmann pers comm) and each female in Hosie's study (1992) tended to mate out of synchrony with the other females, even though they commenced oviposition within five days of each other. Female unresponsiveness during the oviposition period, coupled with spasmodic remating, may be a consequence of physiological constraints on females, such as time taken in oviposition. Each egg takes 5 to 10 minutes to lay, which may ensure fertilisation occurs in the cloaca before the jelly layers hydrate and prevent sperm mobility (Chapter 7). Hosie (1992) has shown that the egg-laying rate of smooth newt females is also dependent on temperature, females may lay up to 60 eggs on warm days in May or June but early in the season they may only lay a few each day (Hosie 1992, pers obs). Thus it may take female smooth newts up to 70 days to lay a full clutch (100 - 600 eggs, Baker 1992a). Smooth newts often breed in temporary ponds, so females may be under selection to devote a large proportion of their time budget to oviposition, to ensure that their full clutch is laid and that the developing larvae have reached metamorphosis before the ponds dry up. So why do females remate during this period, as mating takes time away from oviposition? The findings in chapter 6 suggest that females may remate to replenish their sperm supplies, which may be a consequence of one or more of the following intrinsic female factors:-

a) high utilisation of sperm at the site of fertilisation, due either to the female's inability to regulate the number of sperm released from the spermathecae or to the requirement for a high number of sperm to enter and activate the ovum so that fusion can occur between one sperm and the egg nucleus;

b) limited sperm storage capacity within the spermathecae;

c) sperm leakage from the spermathecae or phagocytosis of sperm within the spermathecae;

d) loss of viability of sperm during storage.

These factors will affect the number of zygotes released by the female and thus, in the absence of remating, may limit her reproductive success.

This study has shown that some females achieved a clutch size in proportion to their body size (estimated from Baker 1992a) after only two spaced matings in a season, suggesting that, in the wild, females may only need to mate a few times to lay their full clutch. Remating in female newts may not be related to body size because, although larger females have a higher requirement for sperm due to higher rates of utilisation and larger clutch sizes than smaller newts (Baker 1992a), females may vary in their capacity to store sperm. Larger individuals have larger organs and glands in many species (Brody 1945, cited in Møller 1988a), so it is likely that the sizes of the spermathecae are also related to female body size. Mating rate is not related to body size in female *Desmognathus ochrophaeus* (Shillington & Verrell 1996), but this may not be comparable to the situation in smooth newts as the females mate multiply before depositing a discrete clutch of eggs.

The low rate of mating by ovipositing females may lead to infrequent mating opportunities. Much of the sexual activity by males is known to be unsuccessful in the wild (Verrell & McCabe 1988; Pavignano *et al.* 1993) and in a semi-natural population, only 5.8% of the courtship encounters resulted in deposition and just 2.2% culminated in sperm transfer (Kauffmann pers comm), suggesting that males are unlikely to deplete their spermatophore supplies sufficiently during a single evening to lower their potential reproductive rate below that of females. The increased availability of sexually active males within the population correspondingly increases the opportunity for female choice. Female smooth newts preferentially pick up sperm from high crested males (Green 1991; Hosie 1992), and sequential mate choice has been shown to occur (Gabor & Halliday 1997). Taken together, these findings suggest that females may use the opportunity of remating to exercise mate choice. Consequently, it is important for males to maintain a good crest and a good spermatophore supply throughout the season, as the supply of receptive females is unpredictable and females are exercising choice based on crest height. Larger males are likely to possess larger crests and to be able to deposit more spermatophores over a longer period than smaller males (chapter 5), suggesting that mating patterns may be influenced by the ratio of larger to smaller males in the population.

Although males in the longitudinal study (chapter 5) deposited fewer spermatophores, each containing lower numbers of sperm, during encounters later in the season compared with earlier encounters, this effect was not seen when males from a natural population were sampled at intervals throughout the season (chapter 4). One explanation for this dichotomy is that 'wild caught' males had achieved fewer matings during the season than individual males tested (with strait-jacketed females) throughout the seasonal study, as mating opportunities in the wild decline with time in season (Verrell & McCabe 1988). Thus, at the end of the season, the 'wild caught' males may have had higher levels of sperm and secretory products than the 'laboratory' males. An alternative interpretation is that the 'wild caught' males were being sampled from successive populations of males. Verrell and Halliday (1985) found that the complete population of smooth newts in a pond in Milton Keynes was only present for one week during the breeding season, so males collected at the end of the season may be males that arrived after the initial migration and missed the opportunity to inseminate large numbers of females. Thus it seems unlikely that males will utilise enough sperm in the wild to be limited by sperm availability during the season, due to the paucity of mating opportunities, unless the population is extremely female biased. Female-biased smooth newt populations are relatively common, ranging from 1.2:1 to 3.6:1 females to males, although these ratios fluctuate from year to year in the same ponds (Griffiths 1996; Arntzen pers comm; Kauffmann pers comm), but the bias is unlikely to be sufficient to prevent the operational sex ratio becoming male-biased fairly quickly as a consequence of individual females mating relatively infrequently. However, even if the seasonal supply of sperm is not limiting male mating capacity, males may be limited by the production of sperm accessory materials. Larger males and males in better reproductive condition may be able to deposit spermatophores for longer in the season, suggesting that males also compete by endurance rivalry. Thus the mating pattern observed in smooth newts may be more accurately classified as scramble competition polygynandry with males competing by endurance rivalry.

In smooth newts, male reproductive success may be constrained, post copulation, by females mating multiply and invoking sperm competition, and by physiological constraints that may be acting on the female, such as limited sperm storage capacity, degradation of sperm during storage and polyspermy. These factors, together with the multiplicity of the spermathecae (40 - 60), may encourage cryptic female choice at all levels within the female tract, e.g. selection of sperm into storage (many sperm are lost in the female tract) and into specific spermathecal tubules, into the fertilisation set (the sperm that are present at the site of fertilisation) and into the egg, and may enable females to continue to exercise mate choice throughout the season. In many species, male seminal fluid contains factors that may influence female reproductive behaviour and physiology (Eberhard & Cordero 1995; Eberhard 1996), which has led Eberhard and Cordero to propose that these male sperm accessory products have evolved through males trying to combat cryptic female choice. Male smooth newts are susceptible to sperm competition (Halliday & Verrell 1984) and cryptic female choice, so it is possible that male newts have also evolved mechanisms or chemicals that influence female reproduction, but these hypotheses await further investigation.

The findings in this thesis, taken together with earlier studies, suggest that male and female physiology and behaviour, in conjunction with ecological factors, such as water temperature, level of resources or length of the breeding season, and social factors, such as mate availability, determine the reproductive strategies of individual males and females and thus determine the mating patterns exhibited by smooth newt populations. These studies highlight the paucity of our knowledge regarding physiological constraints that may be acting on the reproductive mechanisms in male and female smooth newts, and several areas for future research have been identified. These studies have also highlighted the importance of considering factors that may affect both male and female reproductive success in determining the mating patterns of the population; a perspective which has been emphasised previously by West-Eberhard *et al.* (1987). Physiological factors that affect male reproductive potential may also affect the reproductive strategy of their partners

through their need to replenish their sperm supplies. Similarly, the pattern of sperm utilisation in females will have shaped male gametic strategies as they seek to out-compete other males for paternity of the offspring. Selection will favour males that can preempt sperm competition, reduce the likelihood of their own sperm being superseded (Parker 1984) and overcome cryptic female choice (Eberhard 1996). On the other hand, selection will favour females that invoke sperm competition between males and exercise cryptic choice. The question is, which sex will determine the paternity of the offspring? Parker (quoted in Pitnick & Kerr 1996) advocates that the intensity of selection on males to achieve a maximum number of fertilisations is greater than the intensity of selection on females to discriminate among males, thus male advantages in sperm competition will determine paternity of the offspring (Parker 1984). In contrast to this view, Eberhard (1996) proposes that as sperm competition and fertilisation occur within the female tract, females are able to determine paternity through cryptic choice and invoke sperm competition between males to enable them to exercise this choice. As highlighted by Birkhead & Møller (1993), resolving this conflict of paternity is becoming one of the major areas of research today.

8.2. Gametic strategies in other urodeles

This section discusses the gametic strategies exhibited across the families within the superfamily Salamandroidea, in the light of the findings of this study. This section is fairly speculative as data are available for only two species, belonging to different genera within the family Salamandridae, but the hypotheses generated may give valuable insights into the reproductive biology of urodeles and may indicate useful directions for future work.

Newts with internal fertilisation exhibit a variety of modes of courtship, from amplexus throughout the encounter to no contact at all between the male and female, which led Arnold (1977; see also Halliday 1990b; Halliday & Tejedo 1995) to propose that courtship in most urodeles, except *Euproctus* in which the sperm mass is transferred directly into the female's cloaca, may have evolved in response to the unreliability of sperm transfer to a female, via a spermatophore deposited on the substrate. Arnold proposed that courtship

increases the probability of successful insemination of a female and reduces sperm wastage, by decreasing the likelihood that a male will deposit a spermatophore in the absence of a receptive female and by limiting the ability of rival males to interfere in an ongoing mating. The pertinence of these proposals has increased with the findings that sexual interference is widespread throughout the species of urodeles (see review in Halliday & Tejedo 1995), that the seasonal supply of sperm is limited in male newts (see review in Houck & Woodley 1995) and that the production of sperm accessory materials may be constrained physiologically (chapter 3). Also, in the last decade, the focus of attention has shifted to females (Ahnesjö *et al.* 1993). Research in many taxa has shown that females may be determining the paternity of their offspring, via mechanisms that preferentially select sperm for storage or fertilisation (Eberhard 1996). Thus it is timely to consider the gametic strategies of male newts as they seek to maximise their reproductive success under the limitations of finite sperm supplies, competition from rival males for access to females via scramble competition and sexual interference, sperm competition due to females mating multiply, female choice and constraints imposed by female physiology, which may include limited sperm storage capacity, loss of viability of the sperm during storage and polyspermy (multiple sperm entering the ova).

8.2.1. Gametic strategies across the superfamily Salamandroidea

The number of spermatophores that a male deposits during an encounter is a consequence of several opposing selection pressures, such as duration of the breeding season, temporal and spatial patterning of receptive females, mode of courtship, amount of courtship expended per spermatophore and supplies of sperm and sperm accessory materials, that balance the success of mating with a particular female and the likelihood of gaining further matings. In some species the number of spermatophores per courtship correlates well with the length of the breeding season, i.e. males with short breeding seasons (explosive breeders), such as ambystomatids, deposit more spermatophores per encounter than males with longer breeding seasons, such as plethodontids (Arnold 1977; Halliday 1987). However, recent studies have found that some explosively breeding males deposit only one

or two spermatophores during what may be their only mating opportunity for the season, e.g. *Taricha* (Halliday pers comm). These findings suggest that the correlation observed between the number of spermatophores per encounter and the length of the breeding season may be via their mutual correlation with one or more other variables, such as the probability of gaining future matings or the relationship between the number of spermatophores deposited and the likelihood of successful insemination of the female. For example, male *Ambystoma maculatum* deposit a large number of spermatophores during an encounter, but the rate of detection by females is low and sperm wastage is high (Arnold 1977). It is difficult to assess the contribution of gaining future matings as a male may inseminate several females during a single encounter, but the likelihood of females being available on successive evenings may be low (Garton 1972).

At the other extreme, plethodontids, such as *Desmognathus ochrophaeus*, only deposit one or occasionally two spermatophores per encounter, but the certainty of pick-up of each sperm mass by the female is high and sperm wastage is low (Houck *et al.* 1985b; Verrell 1988). Female *D. ochrophaeus* show a propensity to mate multiply in the laboratory (Houck *et al.* 1985b) and multiple paternity occurs in the wild (Tilley & Hausman 1976; Labanick 1983). In between these two extremes lie the salamandrids, such as *T. vulgaris*, which deposit 1 to 5 spermatophores during an encounter. The certainty of pick-up of each sperm mass by the female and the levels of sperm wastage are at intermediate levels between those shown by the ambystomatids and the plethodontids (Halliday 1987). These two factors may influence male gametic strategies, in particular influencing the number of sperm allocated to each sperm mass.

Males of species in which the potential for sperm wastage is high and the certainty of pick-up by the female is low may be predicted to allocate fewer sperm to each sperm mass than males of species in which the reverse is true. Such a prediction would suggest that, compared with *T. vulgaris* males, ambystomatids allocate fewer sperm and plethodontids higher numbers of sperm to each sperm mass, after correcting for male body size. However, this prediction does not take into account the possible effect of sperm

competition on sperm allocation. Males exposed to high levels of sperm competition are predicted to allocate more sperm to their sperm masses than males only exposed to low levels of sperm competition (discussed further in chapter 4). In *T. vulgaris*, the number of females remating in the laboratory within four hours of mating (Arano pers comm) or during a single evening in the wild (Kauffmann pers comm) is low. Thus ambystomatids may allocate higher numbers of sperm to each sperm mass than the number allocated by *T. vulgaris* males because the probability of sperm competition occurring during an evening may be higher. In contrast to female *T. vulgaris*, which may remate within 48 hours of the initial mating, female plethodontids are unresponsive to mating for at least four days after insemination (Verrell 1991b). Thus the likelihood of sperm competition occurring may be low, suggesting that plethodontid males allocate fewer sperm to each sperm mass than *T. vulgaris* males. Which of these possibilities occurs in reality awaits further investigation.

My findings suggest that male *T. vulgaris* mate 'at every available opportunity' (chapter 5), due to the uncertainty of encountering a receptive female. Thus the number of spermatophores deposited by male newts during an encounter may be constrained by the size of their cloacal glands and the rate of replenishment of the cloacal gland secretions after mating (chapter 3). It may be fruitful to examine these parameters in the light of the variation in spermatophore production during an encounter across the superfamily Salamandroidea. My findings suggest that either cloacal gland size or rate of replenishment of the gland secretions or both will be correlated with the number of spermatophores deposited during an encounter. For example, male *Desmognathus ochrophaeus*, which deposit only one spermatophore every two days and produce no spermatophores during the recovery period, may have a slower rate of replenishment and smaller cloacal glands than male *T. vulgaris* (when corrected for body size). Male ambystomatids which deposit a large number of spermatophores (10-81) during one encounter and then deposit relatively few over the next two evenings (20, then 2), may have larger cloacal glands but a slower rate of replenishment compared with male *T. vulgaris*. In fact, as ambystomatids are explosive breeders the males may not replenish the

secretions of the cloacal glands, but may continue to mate until the secretions produced prior to the onset of the breeding season are depleted.

8.2.2. Gametic strategies within the family Salamandridae

The number of sperm per sperm mass has only been measured in one other species, namely, *Taricha torosa* (by myself in Halliday & Hosie in prep). Only one sperm mass from each of five males were available, but these few samples demonstrated that male *T. torosa* allocate between ten and twenty times as many sperm to sperm masses compared with male *T. vulgaris* (Table 8.1).

Table 8.1 Comparison of testes size (as % body weight before and during spermatogenesis) and sperm number per sperm mass.

species	testes weight (corrected for body size)		sperm number (thousands)
Triturus vulgaris	1.5%	6%	38-148
Taricha granulosa	0.3%	2.6%	
Taricha torosa			627-1889

Adult *Taricha species* have a much larger body size (14-15 g compared with 1.9-4.5 g) than adult *T. vulgaris*, so it is predicted that male *Taricha* will allocate more sperm to each sperm mass than *T. vulgaris* males to compensate for dilution effects occurring within the female's larger reproductive tract. However, the testes of male *T. vulgaris* are large for their body size (Verrell *et al.* 1986) compared with the testes of male *Taricha granulosa* (Specker & Moore 1980), suggesting that sperm allocation is a consequence of differing selection pressures acting on the two species. Male *T. torosa* (Davis & Twitty 1964), like male *T. granulosa* (Propper 1991) exhibit amplexus throughout the encounter; the female is released to pick up the sperm mass but then the male engages in amplexus again until the female becomes unresponsive. The breeding season is very short, so unlike adult *T. vulgaris* neither male or female *T. torosa* or *T. granulosa* are likely to mate multiply (Propper 1991). Consequently, in *Taricha species*, the probability of sperm competition occurring is low, the certainty of paternity is high and sperm wastage is low. Thus the

gametic strategy of male *T. torosa* may be to allocate high numbers of sperm to a few spermatophores, as they usually only deposit one spermatophore during an encounter and have few opportunities to mate.

Chapter 4 discussed three strategies which may explain sperm allocation to successive spermatophores. From my studies, it seems unlikely that urodeles allocate sperm in response to the socio-sexual environment. Thus ambystomatids may follow a bet-hedging strategy of depositing spermatophores of similar size and content because the likelihood of a particular sperm mass fertilising any ova is low and the risk of sperm competition is high. Plethodontids may also follow a bet-hedging strategy, but in this case, as may be the situation in *T. vulgaris*, it is to enable males to produce spermatophores throughout a long breeding season. In the family Salamandridae, male *Taricha* are predicted to be following a maximal insemination strategy of depositing a few sperm masses containing high numbers of sperm as they mate infrequently during a very short season. Resolving the gametic strategies of male newts is an interesting area of future study.

References

Ågren, G. (1990). Sperm competition, pregnancy initiation and litter size: influence of the amount of copulatory behaviour in Mongolian gerbils, *Meriones unguiculatus*. *Anim Behav* **40**: 417-427.

Ahnesjö, I., Vincent, A., Alatalo, R., Halliday, T. R. & W. J. Sutherland (1993). The role of females in influencing mating patterns. *Behav Ecol* **4**: 187-189.

Alcock, J., Barrows, E. M., Gordh, G., Hubbard, L. J., Kirkendall, L., Pyle, D. W., Ponder, T. L. & F. G. Zalom (1978). The ecology and evolution of male reproductive behaviour in the bees and wasps. *Zool J Linn Soc* **64**: 293-326.

Andersson, M. (1982). Sexual selection, natural selection and quality advertisement. *Bio J Linn Soc* **17**: 375-393.

Andersson, M. (1986). Evolution of condition dependent sex ornaments and mating preferences: sexual selection based on viability differences. *Evolution* **40**: 804-816.

Andersson, M. (1994). *Sexual Selection*. Princeton: Princeton University Press.

Arak, A. (1988). Sexual dimorphism in body size: A model and a test. *Evolution* **42**: 820-825.

Arnold, S. J. (1977). The evolution of courtship behaviour in new world salamanders with some comments on old world salamanders. In D. H. Taylor & S. I. Guttman (Eds.), *The Reproductive Biology of Amphibians*. New York: Plenum Press. pp141-184.

Arnold, S. J. & T. R. Halliday (1992). Multiple mating by females: the design and interpretation of selection experiments. *Anim Behav* **43**: 178-179.

Arnold, S. J. & M. J. Wade (1984). On the measurement of natural and sexual selection: Applications. *Evolution* **38**: 720-734.

Avery, R. A. (1971). Helminth parasite populations in newts and their tadpoles. *Freshw Biol* **1**: 113-119.

Austin, D. & D. A. Dewsbury (1986). Reproductive capacity of male laboratory rats. *Physiol & Behav* **37**: 627-632.

Baker, J. M. R. (1990a). Body size and spermatophore production in the smooth newt (*Triturus vulgaris*). *Amphibia-Reptilia* **11**: 173-184.

Baker, J. M. R. (1990b). Growth of larval and juvenile newts. Ph.D Thesis. Open University. U.K.

Baker, J. M. R. (1992a). Egg production in the smooth newt (*Triturus vulgaris*). *Herp J* **2**: 90-93.

Baker, J. M. R. (1992b). Body condition and tail height in crested newts, *Triturus cristatus*. *Anim Behav* **43**: 157-159.

Baker, R. R. & M. A. Bellis (1988). 'Kamikaze' sperm in mammals? *Anim Behav* **36**: 936-939.

Baker, R. R. & M. A. Bellis (1989). Elaboration of the kamikaze sperm hypothesis: a reply to Harcourt. *Anim Behav* **37**: 865-867.

Baker, R. R. & M. A. Bellis (1993). Human sperm competition: ejaculate adjustment by males and the function of masturbation. *Anim Behav* **46**: 861-885.

Baker, R. R. & M. A. Bellis (1995). *Human sperm competition. Copulation, masturbation and infidelity.* London: Chapman & Hall.

Bancroft, J. D. & A. Stevens (1990). *Theory and Practice of Histological Techniques. 3rd Edition.* London: Churchill Livingstone.

Barry, F. E., Weatherhead, J. P. & D. P. Philipp (1992). Multiple paternity in a wild population of northern water snakes, *Nerodia sipedon*. *Behav Ecol Sociobiol* **30**: 193-199.

Bateman, A. J. (1948). Intrasexual selection in *Drosophila*. *Heredity* **2**: 349-368.

Baumann, H. (1974). Biological effects of paragonial substances PS1 and PS2 on females of *Drosophila funebris*. *J Insect Physiol* **20**: 2347-2362.

Beach, F. A. (1976). Sexual attractivity, proceptivity and receptivity in female mammals. *Horm Behav* **7**: 105-138.

Bedford, J. M. (1970). The saga of mammalian sperm from ejaculation to syngamy. In H. Gibian & E. J. Plotz (Eds.), *Mammalian Reproduction.* Berlin: Springer. pp 124-182.

Belevedere, P. C., Colombo, L., Mezzalira, F. & G. Malacarne (1984). Steroid biosynthesis by the abdominal gland and the testis of the crested newt, *Triturus cristatus carnifex* Laur. *Laur Boll Zool* **51**: 9.

Bell, G. & J. H. Lawton (1975). The ecology of the eggs and larvae of the smooth newt (*T. vulgaris* (Linn.)). *J Anim Ecol* **44**: 393-424.

Bennett, A. F. & L. D. Houck (1983). The energetic cost of courtship and aggression in a plethodontid salamander. *Ecology* **64**: 979-983.

Birdsall, D. A. & D. Nash (1973). Occurrence of successful multiple insemination of females in natural populations of deer mice (*Peromyscus maniculatus*). *Evolution* **27**: 106-110.

Birkhead, T. R. (1995). Sperm Competition: Evolutionary causes and consequences. *Reprod Fertil Dev* **7**: 755-775.

Birkhead, T. R. (1996). Sperm competition: Evolution and mechanisms. *Curr Top Dev Biol* **33**: 103-158.

Birkhead, T. R., Fletcher, F., Pellatt, E. J. & A. Staples (1995). Ejaculate quality and the success of extra-pair copulations in the zebra finch. *Nature* **377**: 422-423.

Birkhead, T. R. & F. M. Hunter (1990). Mechanisms of sperm competition. *Trends Ecol Evol* **5**: 48-52.

Birkhead, T. R. & A. P. Møller (1992). *Sperm competition in birds: Evolutionary causes and consequences.* London: Academic Press.

Birkhead, T. R. & A. P. Møller (1993). Female control of paternity. *Trends Ecol Evol* **8**: 100-104.

Birkhead, T. R., Møller, A. P. & W. J. Sutherland (1993). Why do females make it so difficult for males to fertilise their eggs? *J theor Biol* **161**: 51-60.

Boisseau, C. & J. Joly (1975). Transport and survival of spermatozoa in female amphibia. In E. S. E. Hafez & C. G. Thibault (Eds.), *The Biology of Spermatozoa: Transport, survival and fertilising ability.* Basel: S Karger. pp94-104.

Bonetti, M. (1996). Geographical variation in growth, size and body condition between amphibian populations. M.Sc. Thesis. The University of Kent at Canterbury, England.

Bradbury, J. W. & M. B. Andersson (1987). (Eds.), *Sexual selection: Testing the alternatives.* Chichester: John Wiley & Sons Ltd.

Brazeau, D. A. & H. R. Lasker (1990). Sexual reproduction and external brooding by the Caribbean gorgonian *Briareum asbestinum. Mar Biol* **104**: 465-474.

Brillard, J. P. & M. R. Bakst (1991). Quantification of spermatozoa in the sperm-storage tubules of turkey hens and its relation to sperm numbers in the perivitelline layer of eggs. *Biol Reprod* **43**: 271-275.

Brizzi, R. & C. Calloni (1992). Male cloacal region of the spotted salamander, *Salamandra salamandra gigliolii* (Amphibia: Salamandridae). *Boll Zool* **59**: 377-385.

Brizzi, R., Calloni, C. & G. Delfino (1990). Accessory structures in the genital apparatus of *Salamandrina terdigitata* (Amphibia: Salamandridae). iv. Male cloacal glands. A study under light and scanning electron microscopes. *Z mikrosk-anat Forsch* **104**: 871-897.

Brizzi, R., Corti, C. & G. Delfino (1995a). Morphology of the dorsal glands in aquatic and terrestrial salamandrids. *8th meeting of SEH* 41.

Brizzi, R., Delfino, G., Selmi M. G. & D. M. Sever (1995b). Spermathecae of *Salamandrina terdigitata* (Amphibia: Salamandridae): Patterns of sperm storage and degradation. *J Morphol* **223**: 21-33.

Brody, S. (1945). *Bioenergetics and growth.* New York: Reinhold.

Capula, M. & L. Luiselli (1994). Can female adders multiply? *Nature* **369**: 528.

Carré, D. & C. Sardet (1984). Fertilisation and early development in *Beroe ovata. Dev Biol* **105**: 188-195.

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F. & L. Partridge (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory products. *Nature* **373**: 241-244.

Charbonneau, M., Moreau, M., Picheral, B., Villain, J. P. & P. Guerrier (1983). Fertilisation of amphibian eggs: a comparison of electrical responses between anurans and urodeles. *Dev Biol* **98**: 304-318.

Cheng, M. (1992). For whom does the female dove coo? A case for the role of vocal self-stimulation. *Anim Behav* **43**: 1035-1044.

Cheng, K. M. & P. B. Siegel (1990). Quantitative genetics of multiple mating. *Anim Behav* **40**: 406-407.

Clutton-Brock, T. H. (1988). (Ed.), *Reproductive Success.* Chicago: The University of Chicago Press.

Clutton-Brock, T. H., Albon, S. D. & F. E. Guinness (1988). Reproductive success in male and female red deer. In T. H. Clutton-Brock (Ed.), *Reproductive Success.* Chicago: The University of Chicago Press.

Clutton-Brock, T. H. (1991). *The Evolution of Parental Care.* Princeton, New York: Princeton University Press.

Clutton-Brock, T. H. & G. A. Parker (1992). Potential reproductive rates and the operation of sexual selection. *Quart Rev Biol* **67**: 437-456.

Clutton-Brock, T. H. & A. C. J. Vincent (1991). Sexual selection and the potential reproductive rates of males and females. *Nature* **351**: 58-60.

Cohen, J. (1973). Crossovers, sperm redundancy and their close association. *Heredity* **31**: 408-413.

Cran, D. G. & C. R. Esper (1990). Cortical granules and the cortical reaction in mammals. *J Reprod Fert Suppl* **42**: 177-188.

Crews, D. (1987). Diversity and evolution of behavioral controlling mechanisms. In D. Crews (Ed.), *Psychobiology of reproductive behavior.* New Jersey: Prentice-Hall Inc. pp88-119.

Davies, N. B. (1985). Cooperation and conflict among dunnocks, *Prunella modularis* in a variable mating system. *Anim Behav* **33**: 628-648.

Davies, N. B. (1991). Mating systems. In J. R. Krebs & N. B. Davies (Eds.), *Behavioural Ecology: An Evolutionary Approach. 3rd Edition.* Oxford: Blackwell Scientific Publications. pp263-300.

Davies, W. C. & V. C. Twitty (1964). Courtship behaviour and reproductive isolation in the species of *Taricha* (Amphibia: Caudata). *Copeia* **1964**: 601-610.

Dent, J. N. (1970). The ultrastructure of the spermatheca in the red-spotted newt. *J Morphol* **132**: 397-423.

Dewsbury, D. A. (1982). Ejaculate cost and mate choice. *Am Nat* **119**: 601-610.

Dewsbury, D. A. (1984). Sperm competition in murid rodents. In R. L. Smith (Ed.), *Sperm competition and the evolution of animal mating systems*. Orlando: Academic press. pp547-571.

Dewsbury, D. A. & D. K. Sawrey (1984). Male capacity as related to sperm production, pregnancy initiation, and sperm competition in deer mice (*Peromyscus maniculatus*). *Behav Ecol Sociobiol* **16**: 37-47.

Diaz-Paniagua, C. (1989). Oviposition behaviour of *Triturus mamoratus pygmaeus*. *J Herpetol* **23**: 159-163.

Dickinson, J. L. (1986). Prolonged mating in the milkweed leaf beetle *Labidomera clivicollis clivicollis* (Coleoptera: Chrysomelidae): A test of the 'sperm-loading' hypothesis. *Behav Ecol Sociobiol* **18**: 331-338.

Dow, J., Lindsay, G. & J. Morrison (1996). *Biochemistry: Molecules, cells and the body*. England: Addison-Wesley Publishers Ltd.

Ducibella, T. (1991). Mammalian cortical granules and the cortical reaction. In P. M. Wassarman (Ed.), *Elements of Mammalian Fertilisation. Vol 1. Basic Concepts.* Boca Raton, Florida, USA: CRC Press, Inc. pp205-231.

Duellman, W. E. & L. Trueb (1994). *Biology of Amphibians.* London: John Hopkins University Press.

Eady, P. E. (1994). Sperm transfer and storage in relation to sperm competition in *Callosobruchus maculatus. Behav Ecol Sociobiol* **35**: 123-129.

Eady, P. E. (1995). Why do male *Callosobruchus maculatus* beetles inseminate so many sperm? *Behav Ecol Sociobiol* **36**: 25-32.

Eberhard, W. G. (1996). *Female control: Sexual selection by cryptic female choice.* New Jersey: Princeton University press.

Eberhard, W. G. & C. Cordero (1995). Sexual selection by cryptic female choice on male seminal products-a new bridge between sexual selection and reproductive physiology. *Trends Ecol Evol* **10**: 493-496.

Elinson, R. P. (1986). Fertilisation in amphibians: The ancestry of the block to polyspermy. *Int Rev Cytol* **101**: 59-100.

Elkan, E (1976). Pathology in the amphibia. In B Lofts (Ed.), *Physiology of the Amphibia. Volume III.* London: Academic Press. pp273-313.

Emlen, S. T. & L. W. Oring (1977). Ecology, sexual selection and the evolution of mating systems. *Science* **197**: 215-223.

Fankhauser, G. (1932). Cytological studies on egg fragments of the salamander Triton. II. The history of the supernumerary sperm nuclei in normal fertilisation and cleavage of fragments containing the egg nucleus. *J Exp Zool* **62**: 185-235.

Fankhauser, G. (1934). Cytological studies, etc. III. The early development of the sperm nuclei in egg fragments without the egg nucleus. *J Exp Zool* **67**: 159-215.

Fankhauser, G. (1948). The organisation of the amphibian egg during fertilisation and cleavage. *Ann N Y Acad Sci* **49**: 684-708.

Fankhauser, G. (1955). The role of the nucleus and cytoplasm. In B. H. Willier, P. A. Weiss & V. Hamburger (Eds.), *Analysis of Development.* London: W. B. Saunders Company. pp126-150.

Fankhauser, G. & C. Moore (1941). Cytological and experimental studies of polyspermy in the newt, *Triturus viridescens*. 1. Normal fertilisation. *J Morphol* **68**: 347-385.

Faria, M. M. (1995). A field study of reproductive interactions in Bosca's newt, *Triturus boscai*. *Amphibia-Reptilia* **16**: 357-374.

Fawcett, D. W. (1970). A comparative view of sperm ultrastructure. *Biol Reprod Suppl* **2**: 90-127.

Fitzpatrick, L. C. (1973). Energy allocation in the Allegheny Mountain salamander, *Desmognathus ochrophaeus*. *Ecol mono* **43**: 43-58.

Forbes, D. J., Kirschner, M. W. & J. W. Newport (1983). Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. *Cell* **34**: 13-23.

Forfanova, K. A. (1964). Morphologic data on polyspermy in chickens. *Zh Obshch Biol* **25**: 22.

Frazer, D. (1989). *Reptiles and Amphibians in Britian.* London: Collins.

Gabor, C. R. (1996). Differential kin discrimination by red-spotted newts (*Notophthalmus viridescens*) and smooth newts (*Triturus vulgaris*). *Ethology* **102**: 649-659.

Gabor, C. R. & T. R. Halliday (1997). Sequential mate choice by multiply mating smooth newts: Females become more choosy. *Behav Ecol* **8**: 162-166.

Gage, M. J. G. (1991). Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Anim Behav* **42**: 1036-1037.

Gage, M. J. G. (1994). Associations between body size, mating pattern, testis size and sperm lengths across butterflies. *Proc R Soc Lond B* **258**: 247-254.

Gage, M. J. G. & R. R. Baker (1991). Ejaculate size varies with sociosexual situation in an insect. *Ecol Entomol* **16**: 331-337.

Gage, A. R. & C. J. Barnard (1996). Male crickets increase sperm number in relation to competition and female size. *Behav Ecol Sociobiol* **38**: 349-353.

Garton, J. S. (1972). Courtship of the small-mouthed salamander, *Ambystoma texanum*, in southern Illinois. *Herpetologica* **28**: 41-45.

Gibbs, H. L.,Weatherhead, P. J., Boag, P. T., White, B. N., Tabak, L. M. & D. J. Hoysak (1990). Realized reproductive success of polygynous red-winged blackbirds revealed by DNA markers. *Science* **250**: 1394-1397.

Gibson, R. M. & P. A. Jewell (1982). Semen quality, female choice, and multiple mating in domestic sheep: A test of Trivers' sexual competence hypothesis. *Behavior* **80**: 9-31.

Gillespie, J. H. (1974). Natural selection for within-generation variance in offspring number. *Genetics* **76**: 601-606.

Gobbetti, A. & M. Zerani (1992). Prostaglandin E_2 and reproduction in the male crested newt, *Triturus carnifex* (Laur). *J Exp Zool* **263**: 186-193.

Green, A. J. (1991). Large male crests, an honest indicator of condition, are preferred by female smooth newts, *Triturus vulgaris* (Salamandridae) at the spermatophore transfer stage. *Anim Behav* **41**: 367-369.

Green, A. J. (1992). Positive allometry is likely with mate choice, competitive display and other functions. *Anim Behav* **43**: 170-172.

Greenberg, N. & J. Wingfield (1987). Stress and reproduction: reciprocal relationships. In D. O. Norris & R. E. Jones (Eds.), *Hormones and Reproduction in Fishes, Amphibians and Reptiles*. London: Plenum Press. pp461-505.

Griffiths, R. A. (1984). Seasonal behaviour and intrahabitat movements in an urban population of smooth newts, *Triturus vulgaris* (Amphibia: Salamandridae). *J Zool Lond* **203**: 241-251.

Griffiths, R. A. (1985). Diel profile of behaviour in the smooth newt, *Triturus vulgaris* (L.): an analysis of environmental cues and endogenous timing. *Anim Behav* **33**: 573-582.

Griffiths, R. A. (1996). *Newts and Salamanders of Europe*. London: T. & A. D. Poyser Ltd.

Griffiths, R. A. & V. J. Mylotte (1988). Observations on the development of the secondary sexual characteristics of male newts *Triturus vulgaris* and *Triturus helveticus*. *J Herpetol* **22**: 476-480.

Gromko, M. H. (1992). Genetic correlation of male and female mating frequency: evidence from *Drosophila melanogaster*. *Anim Behav* **43**: 176-177.

Gromko, M. H., Gilbert, D. G. & R. C. Richmond (1984a). Sperm transfer and use in the multiple mating system of *Drosophila*. In R. L. Smith (Ed.), *Sperm Competition and the Evolution of Animal Mating Systems*. London: Academic Press Inc. pp372-427.

Gromko, M. H., Newport, M. E. & M. G. Kortier (1984b). Sperm dependence of female receptivity to remating in *Drosophila melanogaster*. *Evolution* **38**: 1273-1282.

Gromko, M. H. & T. A. Markow (1993). Courtship and remating in field populations of *Drosophila*. *Anim Behav* **45**: 253-262.

Hagström, T. (1982). Winter habitat selection by some North European amphibians. *Brit J Herpetol* **6**: 276-277.

Haig, D. & C. T. Bergstrom (1995). Multiple mating, sperm competition and meiotic drive. *J Evol Biol* **8**: 265-282.

Halliday, T. R. (1974). Sexual behaviour of the smooth newt, *Triturus vulgaris* (Urodela, Salamandridae). *J Herpetol* **8**: 277-292.

Halliday, T. R. (1975). An observational and experimental study of sexual behaviour in the smooth newt, *Triturus vulgaris*. *Anim Behav* **23**: 291-322.

Halliday, T. R. (1976). The libidinous newt. An analysis of variations in the sexual behaviour of the male smooth newt, *Triturus vulgaris*. *Anim Behav* **24**: 398-414.

Halliday, T. R. (1978). Sexual selection and mate choice. In J. R. Krebs & N. B. Davies (Eds.), *Behavioural Ecology: An Evolutionary Approach. 1st Edition*. Oxford: Blackwell Scientific Publications. pp180-213.

Halliday, T. R. (1980). Sexual behaviour and group cohesion. In H. Kellerman (Ed.), *Group Cohesion: Biological, Psychological and Sociological reflections*. New York: Grune & Stratton. pp171-189.

Halliday, T. R. (1983). The study of mate choice. In P. Bateson (Ed.), *Mate Choice*. Cambridge: Cambridge University Press. pp3-32.

Halliday, T. R. (1987). Physiological constraints on sexual selection. In J. W. Bradbury & M. B. Andersson (Eds.), *Sexual selection: Testing the alternatives*. Chichester: John Wiley & Sons Ltd. pp247-264.

Halliday, T. R. (1990a). Morphology and sexual selection. *Mus Reg Sci Nat Torino* 9-21.

Halliday, T. R. (1990b). The evolution of courtship behaviour in newts and salamanders. *Adv Stud Behav* **19**: 137-169.

Halliday, T. R. (1996). Amphibians. In W. J. Sutherland (Ed.), *Ecological census techniques. A handbook.* Cambridge: Cambridge University Press. pp205-217.

Halliday, T. R. & S. J. Arnold (1987). Multiple mating by females: a perspective from quantitative genetics. *Anim Behav* **35**: 939-941.

Halliday, T. R. & A. I. Houston (1978). The newt as an honest salesman. *Anim Behav* **26**: 1273-1274.

Halliday, T. R. & H. P. A. Sweatman (1976). To breathe or not to breathe; the newts problem. *Anim Behav* **24**: 551-561.

Halliday, T. R. & M. Tejedo (1995). Intrasexual selection and alternative mating behaviour. In H. Heatwole & B. K. Sullivan (Eds.), *Amphibian Biology. Vol 2. Social behaviour*. Chipping Norton, Australia: Surrey Beatty & Sons. pp419-446.

Halliday, T. R. & P. A. Verrell (1984). Sperm competition in amphibians. In R. L. Smith (Ed.), *Sperm Competition and the Evolution of Animal Mating Systems*. New York: Academic Press Inc. pp 487-508.

Halliday, T. R. & P. A. Verrell (1986). Sexual selection and body size in amphibians. *Herp J* **1**: 86-92.

Halliday, T. R. & P. A. Verrell (1988). Body size and age in amphibians and reptiles. *J Herpetol* **22**: 253-265.

Hamilton, W. D. & M. Zuk (1982). Heritable true fitness and bright birds: a role for parasites? *Science* **218**: 384-387.

Hansen, T. F. & D. K. Price (1995). Good genes and old age: Do old mates provide superior genes? *J Evol Biol* **8**: 759-778.

Harcourt, A. H. (1989). Deformed sperm are probably not adaptive. *Anim Behav* **37**: 863-865.

Harcourt, A. H. (1991). Sperm competition and the evolution of non-fertilising sperm in mammals. *Evolution* **45**: 314-328.

Hardy, M. P. & J. N. Dent (1983). A study of the production of spermatophores in the red-spotted newt. *Am Zool* **23**: 973.

Hardy, M. P. & J. N. Dent (1986a). Transport of sperm within the cloaca of the female red-spotted newt. *J Morphol* **190**: 259-270.

Hardy, M. P. & J. N. Dent (1986b). Regulation of motility in sperm of the red-spotted newt. *J Exp Zool* **240**: 385-396.

Hardy, M. P. & J. N. Dent (1987). Hormonal facilitation in the release of sperm from the spermatheca of the red-spotted newt. *Experientia* **43**: 302-304.

Harris, R. N. (1987). An experimental study of population regulation in the salamander *Notophthalmus viridescens dorsalis* (Urodela: Salamandridae). *Oecologia* **71**: 280-285.

Harrison, J. D., Gittins, S. & F. M. Slater (1983). The breeding migrations of smooth and palmate newts (*Triturus vulgaris* and *T. helveticus*) at a pond in mid-Wales. *J Zool Lond* **199**: 249-258.

Harvey, P. H. & P. M. Bennett (1985). Sexual dimorphism and reproductive strategies. In J. Ghesquire, R. D. Martin & F. Newcombe (Eds.), *Human Sexual Dimorphism. Symposia of the society for the study of human biology. Vol 24.* London: Taylor & Francis. pp43-59.

Hasumi, M., Hasegawa, Y. & H. Iwasawa (1993). Long-term maintenance of egg-fertilizability in water of the salamander *Hynobius nigrescens. Jpn J Herpetol* **15**: 71-73.

Hausfater, G., Gerhardt, H. C. & G. M. Klump (1990). Parasites and mate choice in Grey treefrogs, *Hyla versicolor. Am Zool* **30**: 299-311.

Hedlund, L. (1990). Factors affecting differential mating success in male crested newts, *Triturus cristatus. J Zool Lond* **220**: 33-40.

Heidenhain, M. (1890). Beiträge zur Kenntniss der Topographie und Histologie der Kloake und ihrer drusigen Adnexa bei den einheimischen Tritonen. *Arch mikrosk Anat EntwMech* **35**: 173-274.

Hiraizumi, Y. & S. S. Watanabe (1969). Aging effect on the phenomenon of segregation distortion in *Drosophila melanogaster. Genetics* **63**: 121-131.

Ho, S. (1991). Vitellogenesis. In P. K. T. Pang & M. P. Schreibman (Eds.), *Vertebrate Endocrinology: Fundamentals and biomedical implications*. London: Academic Press Ltd. pp91-126.

Hosie, C. A. (1992). Sexual motivation and mate choice in the female smooth newt *T. vulgaris*. Ph.D Thesis. The Open University. U.K.

Houck, L. D. (1988). The effect of body size on male courtship success in a plethodontid salamander. *Anim Behav* **36**: 837-842.

Houck, L. D., Arnold, S. J. & R. A. Thisted (1985b). A statistical study of mate choice: Sexual selection in a plethodontid salamander (*Desmognathus ochrophaeus*). *Evolution* **39**: 370-386.

Houck, L. D. & H. Francillon-Vieillot (1988). Tests for age and size effects on male mating success in a plethodontid salamander. *Amphibia-Reptilia* **9**: 135-144.

Houck, L. D. & D. M. Sever (1994). Role of the skin in reproduction and behaviour. In H. Heatwole & G. T. Barthalmus (Eds.), *Amphibian Biology. Vol 1. The Integument.* Chipping Norton, Australia: Surrey Beatty & Sons. pp351-381.

Houck, L. D., Tilley, S. G. & S. J. Arnold (1985a). Sperm competition in a plethodontid salamander: preliminary results. *J Herpetol* **19**: 420-423.

Houck, L. D. & S. K. Woodley (1995). Field studies of steriod hormones and male reproductive behaviour in amphibians. In H. Heatwole & B. K. Sullivan (Eds.), *Amphibian Biology. Vol 2. Social behaviour.* Chipping Norton, Australia: Surrey Beatty & Sons. pp677-703.

Huck, U. W., Quinn, R. P. & R. D. Lisk (1985). Determinants of mating success in the golden hamster (*Mesocricetus auratus*) IV. Sperm competition. *Behav Ecol Sociobiol* **17**: 239-252.

Humason, G. L. (1979). *Animal Tissue Techniques.* 4th Edition. San Francisco: W. H. Freeman.

Humphrey, R. R. (1977). Factors affecting ovulation in the Mexican axolotl as revealed by induced spawnings. *J Exp Zool* **199**: 209-214.

Iwao, Y. (1989). An electrically mediated block to polyspermy in the primitive urodele *Hynobius nebulosus* and phylogenetic comparison with other amphibians. *Dev Biol* **134**: 438-445.

Iwao, Y. & R. P. Elinson (1990). Control of sperm nuclear behaviour in physiologically polyspermic newt eggs: possible involvement of MPF. *Dev Biol* **142**: 301-312.

Iwao, Y., Sakamoto, N., Takahara, K., Yamashita, M. & Y. Nagahama (1993). The egg nucleus regulates the behaviour of sperm nuclei as well as cycling of MPF in physiologically polyspermic newt eggs. *Dev Biol* **160**: 15-27.

Iwao, Y., Yamasaki, H. & C. Katagiri (1985). Experiments pertaining to the suppression of accessory sperm in fertilised newt eggs. *Dev Growth Differ* **27**: 323-331.

Jaffe, L. A. & M. Gould (1985). Polyspermy-preventing mechanisms. In C. B. Metz & A. Monroy (Eds.), *Biology of fertilisation. Vol 3. The fertilisation response of the egg.* London: Academic Press Inc. pp223-251.

Johnstone, R. A., Reynolds, J. D. & J. C. Deutsch (1996). Mutual mate choice and sex differences in choosiness. *Evolution* **50**: 1382-1391.

Kalezic, M. L., Crnobrnja, J., Dorovic, A. & G. Dzukic (1992). Sexual size difference in *Triturus* newts: geographical variation in Yugoslav populations. *Alytes* **10**: 63-80.

Kaylor, C. T. (1937). Experiments on androgenesis in the newt, *Triturus viridescens*. *J Exp Zool* **76**: 375-394.

Keller, L. & H. K. Reeve (1995). Why do females mate with multiple males? the sexually selected sperm hypothesis. *Adv Stud Behav* **24**: 291-315.

Kikuyama, S., Nakano, R. & I. Yasumasu (1975). Synergistic action of prolactin and androgen on the cloacal glands of the newt. *Comp Biochem Physiol A* **51**: 823-826.

Kikuyama, S., Toyoda, F., Ohmiya, Y., Matsuda, K., Tanaka, S. & H. Hayashi (1995). Sodefrin: A female-attracting peptide pheromone in newt cloacal glands. *Science* **267**: 1643-1645.

King, M. (1990). *Animal Cytogenetics. Vol 4. Chordata 2 Amphibia*. Berlin: Gebrüder Borntraeger.

Knowlton, N. & S. R. Greenwell (1984). Male sperm competition avoidance mechanisms: the influence of female interests. In R. L. Smith (Ed.), *Sperm Competition and the Evolution of Animal Mating Systems.* New York: Academic Press Inc. pp61-84.

Kodric-Brown, A. & J. H. Brown (1984). Truth in advertising: The kind of traits favoured by sexual selection. *Am Nat* **124**: 309-323.

Kuramoto, M. (1995). Scanning electron microscopic studies on the spermatozoa of some Japanese salamanders (Hynobiidae, Crptobranchidae, Salamandridrae). *Jpn J Herpetol* **16**: 49-58.

Kvarnemo, C. & I. Ahnesjo (1996). The dynamics of operational sex ratios and competition for mates. *Trends Ecol Evol* **11**: 404-408.

Labanick, G. (1983). Inheritance of the red-leg and red-cheek traits in the salamander *Desmognathus ochrophaeus*. *Herpetologica* **39**: 114-120.

Labarca, C. & K. Paigen (1980). A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* **102**: 344-352.

Lake, P. E. (1975). Gamete production and the fertile period with particular reference to domesticated birds. *Symp Zool Soc Lond* **35**: 225-244.

Lande, R. (1980). Sexual dimorphism, sexual selection and adaptation in polygenic characters. *Evolution* **34**: 292-305.

Lanier, D. L., Estep, D. Q. & D. A. Dewsbury (1975). Copulatory behaviour of golden hamsters: effects on pregnancy. *Physiol Behav* **15**: 209-212.

Lanier, D. L., Estep, D. Q. & D. A. Dewsbury (1979). Role of prolonged copulatory behaviour in facilitating reproductive success in a competitive mating situation in laboratory rats. *J Comp Physiol Psychol* **93**: 781-792.

Lessells, C. & T. Birkhead (1990). Mechanisms of sperm competition in birds: mathematical models. *Behav Ecol Sociobiol* **27**: 325-337.

Levitan, D. R. (1993). The importance of sperm limitation to the evolution of egg size in marine invertebrates. *Am Nat* **141**: 517-536.

Levitan, D. R. & C. Petersen (1995). Sperm limitation in the sea. *Trends Ecol Evol* **10**: 228-231.

Licht, L. E. & J. P. Bogart (1987). Comparative size of epidermal cell nuclei from shed skin of diploid, triploid and tetraploid salamanders (Genus *Ambystoma*). *Copeia* **1987**: 284-290.

Lodge, J. R., Fechheimer, N. S. & R. G. Japp (1971). The relationship of *in vivo* sperm storage interval to fertility and embryonic survival in the chicken. *Biol Reprod* **5**: 252-257.

Lofts, B. (1974). Reproduction. In Lofts, B. (Ed), *Physiology of the Amphibia. Vol II.* London: Academic Press. pp107-218.

Lofts, B. (1984). Amphibians. In G. E. Lamming (Ed.), *Marshall's physiology of reproduction, Vol 1. Reproductive cycles of vertebrates.* London: Churchill Livingstone. pp127-205.

Lofts, B. (1987). Testicular function. In D. O. Norris & R. E. Jones (Eds.), *Hormones and Reproduction in Fishes, Amphibians and Reptiles.* London: Plenum Press. pp283-326.

Lopez-Leon, M. D., Pardo, M. C., Cabrero, J. & J. P. M. Camacho (1994). Dynamics of sperm storage in the grasshopper, *Eyprepocnemis plorans*. *Physiol Entomol* **19**: 46-50.

Lorenz, L. W. & F. X. Ogasawara (1968). Distribution of spermatozoa in the oviduct and fertility in domestic birds. vi. The relations of fertility and embryo normality with the site of experimental insemination. *J Reprod Fert* **16**: 445-455.

Lorenz, F. W., Bennett, E. B. & M. E. Reynolds (1969). An effect of sperm numbers on abnormal development of avian embryos. *Proc 2nd Ann Meeting Soc Stud Reprod.* Davis.

Madsen, T., Shine, R., Loman, J. & T. Håkansson (1992). Why do female adders copulate so frequently? *Nature* **355**: 440-441.

Malacarne, G. & R. Cortassa (1983). Sexual selection in the crested newt. *Anim Behav* **31**: 1256-1264.

Malacarne, G. & C. Vellano (1987). Behavioral evidence of a courtship pheromone in the crested newt, *Triturus cristatus carnifex* Laurenti. *Copeia* **1987**: 245-247.

Manning, J. T. (1985). Choosy females and correlates of male age. *J theor Biol* **116**: 349-354.

Markow, T. A. (1985). A comparative investigation of the mating system of *Drosophila hydei*. *Anim Behav* **33**: 775-781.

Marks, S. B. & L. D. Houck (1989). Partial cost of spermatophore production in the salamander, *Desmognathus ochrophaeus*. *J Herpetol* **23**: 81-84.

Martin, P. A. & P. J. Dzuik (1977). Assessment of relative fertility of males (cockerels and boars) by competitive mating. *J Reprod Fert* **49**: 323-329.

Martin, P. A., Reimers, T. J., Lodge, J. R. & P. J. Dziuk (1974). The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. *J Reprod Fert* **39**: 251-258.

Massey, A. (1990). Notes on the reproductive ecology of red-spotted newts (*Notophthalmus viridescens*). *J Herpetol* **24**: 106-107.

Matsuda, M. & K. Onitake (1984). Fertilisation of the eggs of *Cynops pyrrhogaster* (Japanese newt) after immersion in water. *Wilhelm Roux's Archives of Developmental Biology* **193**: 61-63.

Mazzi, V. & M. Biciotti (1976). Possible prolactin-testosterone interaction on the cloacal intumescence of the male crested newt. *Atti Accad Sci Torino I Cl Sci Mat Fis Natur* **111**: 75-79.

McLaughlin, E. W. & A. A. Humphries (1978). The jelly envelopes and fertilisation of the eggs of the newt, *Notophthalmus viridescens*. *J Morphol* **158**: 73-90.

Miaud, C. (1993). Predation on newt eggs (*Triturus alpestris* and *T. helveticus*): identification of predators and protective role of oviposition behaviour. *J Zool Lond* **231**: 575-582.

Miaud, C. (1994). Role of wrapping behaviour on egg survival in three species of *Triturus* (Amphibia: Urodela). *Copeia* **1994**: 535-537.

Millar, J. S. & G. J. Hickling (1990). Fasting endurance and the evolution of mammalian body size. *Funct Ecol* **4**: 5-12.

Millar, J. S. & G. J. Hickling (1992). The fasting endurance hypothesis revisited. *Funct Ecol* **6**: 496-498.

Mitchell, S. L. (1990). The mating system genetically affects offspring performance in Woodhouse's toad (*Bufo woodhousei*). *Evolution* **44**: 502-519.

Møller, A. P. (1988a). Ejaculate quality, testis size and sperm competition in primates. *J Human Evol* **17**: 479-488.

Møller, A. P. (1988b). Ejaculate quality, testis size and sperm competition in birds. *Biol J Linn Soc* **33**: 273-283.

Møller, A. P. (1989). Ejaculate quality, testis size and sperm competition in mammals. *Funct Ecol* **3**: 91-96.

Moore, F. L. (1987). Reproductive endocrinology of amphibians. In I. Chester-Jones, P. M. Ingleton & J. G. Phillips (Eds.), *Fundamentals of Comparative Vertebrate Endocrinology*. New York: Plenum Press. pp207-222.

Moore, F. L. & P. Deviche (1988). Neuroendocrine processing of environmental information in amphibians. In M. H. Stetson (Ed.), *Processing of environmental information in vertebrates*. London: Springer-Verlag. pp19-46.

Moore, F. L., McCormack, C. & L. Swanson (1979). Induced ovulation: effects of sexual behaviour and insemination on ovulation and progesterone levels in *Taricha granulosa*. *Gen Comp Endocrinol* **39**: 262-269.

Moore, F. L. & L. J. Miller (1984). Stress-induced inhibition of sexual behaviour: corticosterone inhibits courtship behaviours of a male amphibian (*Taricha granulosa*). *Horm Behav* **18**: 400-410.

Moore, F. L. & R. T. Zoeller (1985). Stress-induced inhibition of reproduction: Evidence of suppressed secretion of LH-RH in an amphibian. *Gen Comp Endocrinol* **60**: 252-258.

Moore, M. C. & C. A. Marler (1988). Hormones, behaviour and the environment: An evolutionary perspective. In M. H. Stetson (Ed), *Processing of environmental information in vertebrates*. London: Springer-Verlag. pp71-84.

Murie, J. O. (1995). Mating behaviour of Columbian ground squirrels. 1. Multiple mating by females and multiple paternity. *Can J Zool* **73**: 1819-1826.

Nakagawa, S. G., Farias, G. J., Suda, D., Cunningham, R. T. & D. L. Chambers (1971). Reproduction of the mediterranean fruit fly: frequency of mating in the laboratory. *Ann Entomol Soc Am* **64**: 949-950.

Nakatsuru, K. & D. L. Kramer (1982). Is sperm cheap? Limited male fertility and female choice in the lemon tetra (Pisces, Characidae). *Science* **216**: 753-755.

Oberhauser, K. S. (1988). Male monarch butterfly spermatophore mass and mating strategies. *Anim Behav* **36**: 1384-1388.

Ogasawara, F. X. & F. W. Lorenz (1966). Early mortality in chick embryos following intramagnal insemination. *Poult Sci* **45**: 1112.

Olsson, M., Gullberg, A., Tegelström, H., Madsen, T. & R. Shine (1994). Can female adders multiply? *Nature* **369**: 528.

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biol Rev* **45**: 525-567.

Parker, G. A. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *J theor Biol* **96**: 281-294.

Parker, G. A. (1984). Sperm competition and the evolution of animal mating strategies. In R. L. Smith (Ed.), *Sperm Competition and the Evolution of Animal Mating Systems*. New York: Academic press Inc. pp1-60.

Parker, G. A. (1990). Sperm competition games: raffles and roles. *Proc R Soc Lond B* **242**: 120-126.

Parker, G. A. (1992). Snakes and female sexuality. *Evol Biol* **355**: 395-396.

Pavignano, I., Sacchetto, C. & C. Giacoma (1993). Field observations of the sexual behaviour of the smooth newt, *Triturus vulgaris*. *Ethol Ecol Evol* **5**: 406-407.

Pecio, A. (1992). Insemination and egg-laying dynamics in the smooth newt, *Triturus vulgaris*, in the laboratory. *Herp J* **2**: 5-7.

Pellett, E. J. & T. R. Birkhead (1994). Ejaculate size in zebra finches *Taeniopygia guttata* and a method for obtaining ejaculates from passerine birds. *Ibis* **136**: 97-101.

Philippi, T. & J. Seger (1989). Hedging one's evolutionary bets, revisited. *Trends Ecol Evol* **4**: 41-44.

Picheral, B. (1977). La fecondation chez le triton, Pleurodele. I. la traversée des enveloppes de l'oeuf par les spermatozoides. *J Ultrastr Res* **60**: 106-120.

Picheral, B. (1979). Structural, comparative, and functional aspects of spermatozoa in urodeles. In D. W. Fawcett & J. M. Bedford (Eds.), *The Spermatozoon*. Baltimore-Munich: Urban & Schwarzenberg Inc. pp267-287.

Pierce, J. D. J., Ferguson, B., Salo, A. L., Sawrey, D. K., Shapiro, L. E., Taylor, S. A. & D. A. Dewsbury (1990). Patterns of sperm allocation across successive ejaculates in four species of voles (*Microtus*). *J Reprod Fert* **88**: 141-149.

Pitnick, S. & T. Karr (1996). Sperm Caucus. *Trends Ecol Evol* **11**: 148-151.

Pitnick, S. & T. A. Markow (1994). Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. *Am Nat* **143**: 785-819.

Propper, C. R. (1991). Courtship in the rough-skinned newt, *Taricha granulosa*. *Anim Behav* **41**: 547-554.

Propper, C. R. & F. L. Moore (1991). Effects of courtship on brain gonadotropin hormone-releasing hormone and plasma steroid concentrations in a female amphibian (*Taricha granulosa*). *Gen Comp Endocrinol* **81**: 304-312.

Pyle, D. W. & M. H. Gromko (1978). Repeated mating by female *Drosophila melanogaster*: The adaptive importance. *Experientia* **34**: 449-450.

Rafinski, J. (1981). Multiple paternity in natural populations of the alpine newt, *Triturus alpestris* (Laur). *Amphibia-Reptilia* **2**: 282.

Raxworthy, C. J. (1989). Courtship, fighting and sexual dimorphism of the banded newt *Triturus vittatus ophryticus*. *Ethology* **81**: 148-170.

Ridley, M. (1989). The incidence of sperm displacement in insects: four conjectures, one corroboration. *Biol J Linn Soc* **38**: 349-367.

Reynolds, J. D. (1996). Animal breeding systems. *Trends Ecol Evol* **11**: 68-72.

Robin, C. (1874). Observations sur la fécondation des urodeles. *J Anat Physiol Norm Path* **10**: 376-390.

Romo, E., De Miguel, M. P., Arenas, M. I., Frago, L., Fraile, B. & R. Paniagua (1996). Histochemical and quantitative study of cloacal glands of *Triturus marmoratus marmoratus* (Amphibia: Salamandridae). *J Zool Lond B* **239**:177-186.

Rusconi, M. (1821). *Amours des Salamandres aquatiques*. Milan.

Rutowski, R. L. (1979). The butterfly as an honest salesman. *Anim Behav* **27**: 1269-1270.

Sakaluk, S. K. & W. H. Cade (1980). Female mating frequency and progeny production in singly and doubly mated house and field crickets. *Can J Zool* **58**: 404-411.

Seger, J. & H. J. Brockmann (1987). What is bet-hedging? *Oxford surveys in evolutionary biology* **4**: 182-211.

Semik, D. & W. Kilarski (1986). Surface morphology of eggs and early embryos of three species of newts. *Z mikrosk-anat forsch Leipzig* **100**: 881-904.

Semlitsch, R. D. (1994). Evolutionary consequences of non-random mating: do large males increase offspring fitness in the anuran *Bufo bufo*? *Behav Ecol Sociobiol* **34**: 19-24.

Semlitsch, R. D., Scott, D. E. & J. H. K. Pechman (1988). Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* **69**: 184-192.

Sever, D. M. (1981). Cloacal anatomy of male salamanders in the families Ambystomatidae, Salamandridae, and Plethodontidae. *Herpetologica* **37**: 142-155.

Sever, D. M. (1991). Sperm storage and degradation in the spermathecae of the salamander, *Eurycea cirrigera*. *J Morphol* **210**: 71-84.

Sever, D. M. (1992). Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). iv Salamandridae. *Anat Rec* **232**: 229-244.

Sever, D. M. (1994a). Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). vii Plethodontidae. *IIerp Monographs* **8**: 276-337.

Sever, D. M. (1994b). Observations on regionalization of secretory activity in the spermathecae of salamanders and comments on phylogeny of sperm storage in female amphibians. *Herpetologica* **50**: 383-397.

Sever, D. M. & N. S. Brunette (1993). Regionalization of eccrine and spermiophagic activity in spermathecae of the salamander *Eurycea cirrigera* (Amphibia: Plethodontidae). *J Morphol* **217**: 161-170.

Sever, D. M. & L. Houck (1985). Spermatophore formation in *Desmognathus ochrophaeus* (Amphibia: Plethodontidae). *Copeia* **1985**: 394-402.

Sever, D. M. & N. M. Kloepfer (1993). Spermathecal cytology of *Ambystoma opacum* (Amphibia: Ambystomatidae) and the phylogeny of sperm storage organs in female salamanders. *J Morphol* **217**: 115-127.

Sever, D. M., Rania, L. C. & J. D. Krenz (1996). Annual cycle of sperm storage in the spermathecae of the red-spotted newt, *Notophthalmus viridescens* (Amphibia: Salamandridae). *J Morphol* **227**: 155-170.

Sever, D. M., Verrell, P. A., Halliday, T. R., Griffiths, M. & V. Waights (1990). The cloaca and cloacal glands of the male smooth newt *Triturus vulgaris vulgaris* (Linneaus) with especial emphasis on the dorsal gland. *Herpetologica* **46**: 160-168.

Shapiro, D. Y., Marconato, A. & T. Yoshikawa (1994). Sperm economy in a coral reef fish, *Thalassoma bifasciatum*. *Ecology* **75**: 1334-1344.

Sherman, P. W. & D. F. Westneat (1988). Multiple mating and quantitative genetics. *Anim Behav* **36**: 1545-1547.

Shillington, C. & P. A. Verrell (1996). Multiple mating by females is not dependent on body size in the salamander *Desmognathus ochrophaeus*. *Amphibia-Reptilia* **17**: 33-38.

Simmons, L. W. (1990). Nuptial feeding in tettigoniids: male costs and the rates of fecundity increase. *Behav Ecol Sociobiol* **27**: 43-47.

Simmons, L. W. (1995). Male bush crickets tailor spermatophores in relation to their remating intervals. *Funct Ecol* **9**: 881-886.

Simmons, L.W., Stockley, P., Jackson, R. L. & G. A. Parker (1996). Sperm competition or sperm selection: no evidence for female influence over paternity in yellow dung flies *Scatophaga stercoraria*. *Behav Ecol Sociobiol* **38**: 199-206.

Simmons, L. W., Teale, R. J., Maier, M., Standish, R. J., Bailey, W. & P. C. Withers (1992). Some costs of reproduction for male bushcrickets *Requena verticalis* (Orthoptera: Tettigoniidae): allocating resources to mate attraction and nuptial feeding. *Behav Ecol Sociobiol* **31**: 57-62.

Sivinski, J. (1984). Sperm in competition. In R. L. Smith (Ed.), *Sperm Competition and the Evolution of Animal Mating Systems*. London: Academic Press. pp86-116.

Slack, J. M. W. (1983). *From Egg to Embryo: Determinative events in early development.* Cambridge: Cambridge University Press.

Slatkin, M. (1974). Hedging one's evolutionary bets. *Nature* (*London*) **250**: 704-705.

Smith, M. (1951). *The British Amphibians & Reptiles.* London: Collins.

Smith, C., King, J. W. B. & N. Gilbert (1962). Genetic parameters of British large white bacon pigs. *Anim Prod* **4**: 128-143.

Smith-Gill, S. J. & K. A. Berven (1979). Predicting amphibian metamorphosis. *Am Nat* **113**: 563-585.

Snook, R. R., Markow, T. A. & T. L. Karr (1994). Functional nonequivalence of sperm in *Drosophilia pseudoobscura. Proc Natl Acad Sci USA* **91**: 11222-11226.

Spallanzani, L. (1780). Dissertazioni di fisica animale e vegetabile. Modena.

Sparreboom, M. (1984). Gedrag en voortplanting van de wrattensalamanders *Paramesotriton honkongensis* en P. *chinensis. Lacerta* **43**: 28-35.

Speakman, J. R. (1992). Evolution of animal body size: a cautionary note on assessments of the role of energetics. *Funct Ecol* **6**: 495-496.

Specker, J. L. & F. Moore (1980). Annual cycle of plasma androgens and testicular composition in the rough-skinned newt, *Taricha granulosa. Gen Comp Endocrinol* **42**: 297-303.

Stearns, S. C. (1992). *The evolution of life histories.* Oxford: Oxford University Press.

Streett, J. C. (1940). Experiments on the organisation of the unsegmented egg of *Triturus pyrrhogaster. J Exp Zool* **85**: 383-408.

Suarez, S. S., Drost, M., Redfern, K. & W. Gottlieb (1990). Sperm motility in the oviduct. In B. D. Bavister, J. Cummins & E. R. S. Roldan (Eds). *Fertilisation in mammals.* Serono Symposia, Norwell, Mass. pp111-124.

Sullivan, B. K., Ryan, M. J. & P. A. Verrell (1995). Female choice and mating system structure. In H. Heatwole & B. K. Sullivan (Eds.), *Amphibian Biology. Vol 2. Social behaviour.* Chipping Norton, Australia: Surrey Beatty & Sons. pp469-517.

Svärd, L. (1985). Parental investment in a monandrous butterfly, *Pararge aegeria*. *Oikos* **45**: 66-70.

Svärd, L. & C. Wiklund (1986). Different ejaculate delivery strategies in first versus subsequent matings in the swallowtail butterfly *Papilio machaon* L. *Behav Ecol Sociobiol* **18**: 325-330.

Svärd, L. & C. Wiklund (1989). Mass and production rate of ejaculates in relation to monandry/polyandry in butterflies. *Behav Ecol Sociobiol* **24**: 395-402.

Svärd, L. & C. Wiklund (1991). The effect of ejaculate mass on female reproductive output in the European swallowtail butterfly *Papilio machaon* (L.) (Lepidoptera: Papilionidae). *J Insect Behav* **4**: 33-41.

Taylor, O. R. (1967). Relationship of multiple mating to fertility in *Atteva punctella* (Lepidoptera: Yponomeutidae). *Ann Entomol Soc Am* **60**: 583-590.

Teyssedre, C. & T. Halliday (1986). Cummulative effect of male's displays in the sexual behaviour of the smooth newt *Triturus vulgaris* (Urodela, Salamandridae). *Ethology* **71**: 89-102.

Thiesmeier, B. & C. Hornberg (1990). Zur Fortpflanzung sowie zum Paarungsverhalten der Gebirgsmolche, Gattung *Euproctus* (Gené), im Terrarium, unter besonderer Berücksichtigung von *Euproctus asper* (Duges, 1852). *Salamandra* **26**: 63-82.

Thornhill, R. & J. Alcock (1983). *The Evolution of Insect Mating Systems.* Cambridge, Massachusetts: Harvard University Press.

Thrall, P. H., Antonovics, J. & J. D. Bever (1997). Sexual transmission of disease and host mating systems. I. Within-season reproductive success. *Am Nat* **149**: 485-506.

Tilley, S. G. & J. S. Hausman (1976). Allozymic variation and occurrence of multiple inseminations in populations of the salamander *Desmognathus ochrophaeus*. *Copeia* **1976**: 734-741.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed.), *Sexual selection and the descent of man 1871-1971*. Chicago: Aldine. pp136-179.

Uzzell (1969). Notes on spermatophore production by salamanders of the *Ambystoma jeffersonianum* complex. *Copeia* **1969**: 602-612.

Vahed, K. & F. S. Gilbert (1996). Differences across taxa in nuptial gift size correlate with differences in sperm number and ejaculate volume in bushcrickets (Orthoptera: Tettigoniidae). *Proc R Soc Lond B* **263**: 1257-1265.

Vehrencamp, S. L. & J. W. Bradbury (1984). Mating systems and ecology. In J. R. Krebs & N. B. Davies (Eds.), *Behavioural Ecology: An Evolutionary approach. 2nd Edition.* Oxford: Blackwell Scientific Publications. pp251-278.

Veith, M. (1987). Weight condition in *Triturus alpestris*-methological and ecological aspects. *Proc 4th Ord Meet SEH* 429-432.

Vellano, C., Mazzi, V. & M. Sacerdote (1970). Tail height, a prolactin-dependent ambisexual character in the newt (*Triturus cristatus carnifex* Laur.). *Gen Comp Endocrinol* **14**: 535-541.

Verrell, P. A. (1982a). Male newts prefer large females as mates. *Anim Behav* **30**: 1254-1255.

Verrell, P. A. (1982b). The sexual behaviour of the red-spotted newt *Notophthalmus viridescens* (Amphibia: Urodela: Salamandridae). *Anim Behav* **30**: 1224-1236.

Verrell P. A. (1982c). A note on the maintenance of the red-spotted newt in captivity. *Brit Herp Soc Bull* **5** 28.

Verrell, P. A. (1984a). Sexual interference and sexual defense in the smooth newt *Triturus vulgaris* (Amphibia: Urodela: Salamandridae). *Z Tierspsychol* **66**: 242-254.

Verrell, P. A. (1984b). The responses of inseminated female smooth newts *Triturus vulgaris* to further exposure to males. *Brit J Herpetol* **6**: 414-415.

Verrell, P. A. (1984c). Egg extrusion afer belly pressing: a simple means for the determination of ovulation in the smooth newt. *Herp Rev* **15**: 39-40.

Verrell, P. A. (1985). The winter habitats of amphibians in Milton Keynes, England. *Brit Herp Soc Bull* **13**: 24-27.

Verrell, P. A. (1986a). Limited male mating capacity in the smooth newt, *Triturus vulgaris vulgaris* (Amphibia). *J Comp Psychol* **100**: 291-295.

Verrell, P.A. (1986b). Wrestling in the red-spotted newt (*Notophthalmus viridescens*): resource value and contestant asymmetry determine contest duration and outcome. *Anim Behav* **34**: 398-402.

Verrell, P. A. (1986c). Male discrimination of larger, more fecund females in the smooth newt, *Triturus vulgaris*. *J Herpetol* **20**: 416-422.

Verrell, P. A. (1988). Intrinsic male mating capacity is limited in the plethodontid salamander, *Desmognathus ochrophaeus*. *J Herpetol* **22**: 394-400.

Verrell, P. A. (1989). The sexual strategies of natural populations of newts and salamanders. *Herpetologica* **45**: 265-282.

Verrell, P. A. (1991a). Proximity preferences in female smooth newts, *Triturus vulgaris vulgaris*. Do they reveal complex mating decisions? *Copeia* **1991**: 835-836.

Verrell, P. A. (1991b). Insemination temporarily inhibits sexual responsiveness in female salamanders (*Desmognathus ochrophaeus*). *Behaviour* **119**: 51-64.

Verrell, P. A. & A. Donovan (1991). Male-male agression in the plethodontid salamander, *Desmognathus ochrophaeus*. *J Zool Lond* **223**: 203-212.

Verrell, P. A. & H. Francillon (1986). Body size, age and reproduction in the smooth newt, *Triturus vulgaris*. *J Zool Lond A* **210**: 89-100.

Verrell, P. A. & T. R. Halliday (1985). Reproductive dynamics of a population of smooth newts, *Triturus vulgaris*, in Southern England. *Herpetologica* **41**: 386-395.

Verrell, P. A., Halliday, T. R. & M. L. Griffiths (1986). The annual reproductive cycle of the smooth newt (*Triturus vulgaris*) in England. *J Zool Lond A* **210**: 101-119.

Verrell, P. A. & N. McCabe (1988). Field observations of the sexual behaviour of the smooth newt, *Triturus vulgaris vulgaris* (Amphibia: Salamandridae). *J Zool Lond* **214**: 533-545.

Verrell, P. A. & D. M. Sever (1988). The cloaca and spermatheca of the female smooth newt, *Triturus vulgaris* L. (Amphibia: Urodela: Salamandridae). *Acta Zool (Stockholm)* **69**: 65-70.

Wahlert, G. (1953). Eileiter, Laich und Kloake der Salamandriden. *Zool Jb* **73**: 276-324.

Waights, V. (1996). Female sexual interference in the smooth newt *Triturus vulgaris vulgaris*. *Ethology* **102**: 736-747.

Wakimoto, B. T. (1979). DNA synthesis after polyspermic fertilisation in the axolotl. *J Embryol Exp Morph* **52**: 39-48.

Walker, W. F. (1980). Sperm utilisation strategies in non-social insects. *Am Nat* **115**: 780-799.

Ward, P. (1993). Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria*. *Behav Ecol Sociobiol* **32**: 313-319.

Wells, K. D. & T. L. Taigen (1986). The effect of social interactions on calling energetics in the gray treefrog (*Hyla versicolor*). *Behav Ecol Sociobiol* **19**: 9-18.

West-Eberhard, M. J. *et al* (1987). Conflicts between and within the sexes in sexual selection, group report. In J. W. Bradbury &. M. B. Andersson (Eds.), *Sexual selection: Testing the alternatives*. Chichester: John Wiley & sons Ltd. pp247-264.

Whittier, J. M. (1991). Artificial regulation of reproductive cycles. In P. K. T. Pang & M. P. Schreibman (Eds.), *Vertebrate endocrinology: Fundamentals and biomedical implications*. London: Academic Press Ltd. pp243-268.

Wiklund, C. & A. Kaitala (1995). Sexual selection for large male size in a polyandrous butterfly: the effect of body size on male versus female reproductive success in *Pieris napi*. *Behav Ecol* **6**: 6-13.

Williams, G. C. (1966). *Adaptation and natural selection.* Princeton: Princeton University Press.

Williams, G. C. (1978). Mysteries of sex and recombination. *Quart Rev Biol* **53**: 287-289.

Wimpenny, R. S. (1951). The effect of vegetation on the breeding of newts, *Molge cristata* and *Molge vulgaris*. *J Anim Ecol* **20**: 98-100.

Wilson, J. R., Adler, N. & B. Leboeuf (1965). The effects of intromission frequency on successful pregnancy in the female rat. *Proc Natl Acad Sci USA* **53**: 1392-1395.

Wingfield, J. C. & G. J. Kenagy (1991). Natural regulation of reproductive cycles. In P. K. T. Pang & M. P. Schreibman (Eds.), *Vertebrate endocrinology: Fundamentals and biomedical implications*. London: Academic Press Ltd. pp181-242.

Woodward, B. D. (1987). Paternal effects on offspring traits in *Scaphiopus couchi* (Anura: Pelobatidae). *Oecologia* **73**: 626-629.

Woodward, B. D. & J. Travis (1991). Paternal effects on juvenile growth and survival in spring peepers (*Hyla crucifer*). *Evol Ecol* **5**: 40-51.

Wortham, J. W. E., Muphy, J. A., Martan, J. & R. A. Brandon (1982). Scanning electron microscopy of some salamander spermatozoa. *Copeia* **1982**: 52-60.

Wyrick, R. E., Nishihara, T. & J. L. Hedrick (1974). Agglutination of jelly coat and cortical granule components and the block to polyspermy in the amphibian *Xenopus laevis*. *Proc Natl Acad Sci USA* **71**: 2067-2071.

Yamamoto, K., Toyoda, F., Tanaka, S., Hayashi, H. & S. Kikuyama (1996). Radioimmunoassay of a newt sex pheromone, sodefrin, and the influence of hormones on its level in the abdominal gland. *Gen Comp Endocrinol* **104**: 356-363.

Yuval, B., Blay, S. & R. Kaspi (1996). Sperm transfer and storage in the mediterranean fruit fly (Diptera: Tephritidae). *Ann Entomol Soc Am* **89**: 486-492.

Zahavi, A. (1975). Mate selection: a selection for a handicap. *J theor Biol* **53**: 205-214.

Zahavi, A. (1977). The cost of honesty (Further remarks on the handicap principle). *J theor Biol* **67**: 603-605.

Zalisko, E. J., Brandon, R. A. & J. Martan (1984). Microstructure and histochemistry of salamander spermatophores (Ambystomatidae, Salamandridae and Plethodontidae). *Copeia* **1984**: 739-737.

Zeller, E. (1905). Untersuchungen über die Samenträger und den Kloakenwulst der Tritonen. Zeitsch. Wissen. *Zool* **74**: 171-221.

Zerani, M. & A. Gobbetti (1993). Corticosterone during the annual reproductive cycle and in the sexual behaviour in the crested newt *Triturus carnifex*. *Horm Behav* **27**: 29-37.

Zerani, M., Vellano, C., Amabili, F., Carnevali, O., Andreoletti, G. E. & A. Polzonetti-Magni (1991). Sex steroid profile and plasma vitellogenin during the annual reproductive cycle of the crested newt (*Triturus carnifex* Laur.). *Gen Comp Endocrinol* **82**: 337-344.